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PLANT PHYSIOLOGY

VOLUME 27 JULY, 1952 NUMBER 3

THE UPTAKE OF PHOSPHORUS BY BEAN PLANTS WITH PARTICULAR REFERENCE TO THE EFFECTS OF IRON

O. Biddulph and C. G. Woodbridge

(WITH FOUR FIGURES)

Received September 21, 1951

This paper presents some of the findings of a study of the uptake of phosphorus by bean plants as it is influenced by iron. As a result of this work a number of points have been brought out which have not been clearly stated previously. In order to complete the study successfully, the effects of pH and the simultaneous effects of various phosphorus and iron supplies were considered. The results show: (a) Absorbed iron ties up a portion of the "seed phosphorus" in an unusable condition. (b) A ferric phosphate precipitate, in or on the roots, retards the flow of phosphorus to the actively growing leaves. (c) As the phosphorus content of the nutrient medium is increased, roots, stems, and cordate leaves continue to build up in phosphorus content even after trifoliate leaves are being adequately supplied. The excess phosphorus may be responsible for immobilizing iron and other ions.

The effect of the hydrogen ion concentration on the absorption of phosphorus by higher plants was studied by Arnon *et al.* (**2**) as part of a more comprehensive investigation of the absorption of inorganic nutrients. They showed that the amount of phosphate absorbed varied both with the plant and also with the pH of the nutrient solution. With tomato, maximum absorption occurred at pH 7, decreasing toward pH 3 and pH 9. With Bermuda grass there was no marked variation in the phosphorus absorption between pH 4 and pH 7, although a tendency toward a minimum appeared at pH 6. At a pH of 3 and pH 9, which are extremes under which plants barely persist, the phosphorus absorption was much lower. Because of this variation between plants, a study was undertaken of the absorption of phosphorus by bean plants grown in water cultures maintained at various pH values.

The effect of iron on the absorption of phosphorus has not been studied directly. However, relationships between the two have been reported by a number of workers. Aiyar (**1**), using solution cultures, found that increasing concentrations of phosphorus caused an increase in the phosphorus content of the roots, but a decrease in the nitrogen and iron contents. Thomas

et al. (**14**) showed that phosphatic fertilizer reduced the absorption of iron by *Calluna vulgaris*. SIDERIS *et al.* (**11**) reported that in *Ananas comosus* an increased supply of phosphorus increased slightly the amount of iron precipitated on the plant's roots. These papers, while dealing more or less indirectly with the effect of phosphorus on the absorption of iron, do not present a thorough elucidation of the phenomenon.

The relationship of phosphorus to chlorosis has been the subject of considerable study and a few papers have contained information of direct bearing on it. OLSEN (**8, 9**) demonstrated that certain plants (*Lemna polythiza*, *Zea mays*, and *Xanthium spinosum*), when grown in water culture solutions (Knop's solution at pH 7.0) having a high phosphate content but normal iron content, became chlorotic even though the iron content of the chlorotic leaves compared favorably with that present in green leaves. WADLEIGH *et al.* (**15**) grew corn plants in solutions high in phosphate and found that these plants were also severely chlorotic. FRANCO and LOOMIS (**6**) compared the absorption of phosphorus and iron from various nutrient solutions and found that true iron-deficiency chlorosis could be prevented if phosphorus was withheld for two to four days from the nutrient solution at the start of an experiment.

Methods

Red kidney bean seeds (*Phaseolus vulgaris*) were dusted with Semesan (active fungicidal ingredient, hydroxymercurichlorophenol) and placed between dampened paper towelling in a shallow glass dish where they remained for two days. The radicles from viable seeds were then evident, and the sprouted seeds were transferred to glass beads partially immersed in tap water. The seedlings remained on the glass beads for five to six days. During this time, the hypocotyls grew sufficiently so that the young bean plants could be transferred to half-strength nutrient solution contained in enamelled pans of six liters capacity. The plants were supported by wrapping non-absorbent cotton about the stems and inserting them into slots cut in Lucite covers. Covers were painted with black asphaltum varnish on the lower surface and with white paint on the upper surface. Each pan contained six plants. The day of transfer to the pans was always designated as zero day, and periods in solution and age of plants were calculated from this base. The solutions were changed to full-strength on the fourth day and changed again on the eighth day. Harvests were made on the twelfth day unless stated otherwise.

The nutrient solutions were varied with respect to their phosphorus content, iron content, and pH. The variations are given with each experiment. The pH of each solution was adjusted twice daily (early morning and late afternoon) by the addition of potassium hydroxide solution or sulphuric acid solution. The basic nutrient medium had the following composition:

$Ca(NO_3)_2$—0.0025 M	H_3BO_3—0.5 p.p.m. B
KNO_3 —0.0025 M	$CuSO_4$—0.02 p.p.m. Cu

$MgSO_4$ —0.001 M	$MnCl_2$—0.5 p.p.m. Mn
KH_2PO_4 —0.00025 M (except as varied)	MoO_3 —0.01 p.p.m. Mo
$Fe(NO_3)_3$—1.0 p.p.m. Fe (except as varied)	$ZnSO_4$—0.05 p.p.m. Zn

The solutions were continuously aerated by means of carbon pipe aerators and compressed air at three pounds pressure. Sunlight was supplemented during the winter months with daylight fluorescent lamps, but the intensity was still relatively low as compared to sunlight.

Leaves were harvested according to position upon the stem. The petioles were considered as part of the stem. Tissues were dried at 80° C to constant weight, ground, and the aliquots weighed for analysis.

An aliquot of five mg. of dried tissues was used for analysis for total phosphorus (P^{31}). This amount was transferred to a 10-ml. Pyrex volumetric flask and was digested in one ml. of 5 *N* sulphuric acid solution and one drop of 60% perchloric acid solution. A sand bath was used, and about three hours were required for the digestion. After the solution had cleared, the flask was cooled, and 7 ml. of water were added followed by the reagents for development of the well-known phosphomolybdate blue (**12**). The intensity of the color was measured in a photelometer using a red (610–P) filter and was compared with standards prepared at the same time.

When radiophosphorus was to be determined, an aliquot (20 to 100 mg.) of dried material was weighed and transferred to a 30-ml. porcelain crucible. The sample was ashed directly in a muffle furnace at dull red heat and then cooled. The ash was dissolved in dilute hydrochloric acid to insure uniform spreading in the crucible. The solution was evaporated to dryness and counted. The geometry factor was constant for all determinations. Errors due to self-absorption, coincidence phenomena, and counter efficiency were low in comparison to the statistical errors of counting and were ignored. The quantity of radiophosphorus used in each experiment was calculated so that the net number of counts in the weakest sample would exceed twice the background. Statistical examination of the counting procedure (**7**) indicates that the probable error of the results is not greater than 10% in cases of low activity and in the order of 5% for higher activity.

Results and discussion

The effect of pH upon the absorption of phosphorus

It is known that the hydrogen ion concentration of a nutrient solution exerts an effect both upon the nutrient solution and upon the roots of plants growing in it. Within the nutrient solution the form of the phosphate ion is determined by the pH, with $H_2PO_4^-$ predominating below approximately 6.7 and $HPO_4^=$ above that value (fig. 1). The hydrogen ion concentration also exerts an effect upon the permeability of the membranes of the absorbing cells. This is a complex phenomenon, and its discussion will not be attempted here. As it has been shown by Arnon *et al.* (**2**) that pH influences phosphorus absorption differently in different plants, it was necessary to determine its effect on phosphorus absorption by bean plants.

The problem of phosphorus absorption at different pH values was investigated by measuring the uptake and the resultant distribution of this element between the various plant organs. The pH values employed were 4, 5, 6, and 7, each under conditions of no iron supplementation and under conditions wherein the solutions contained 1 p.p.m. Fe (0.000018 M). The

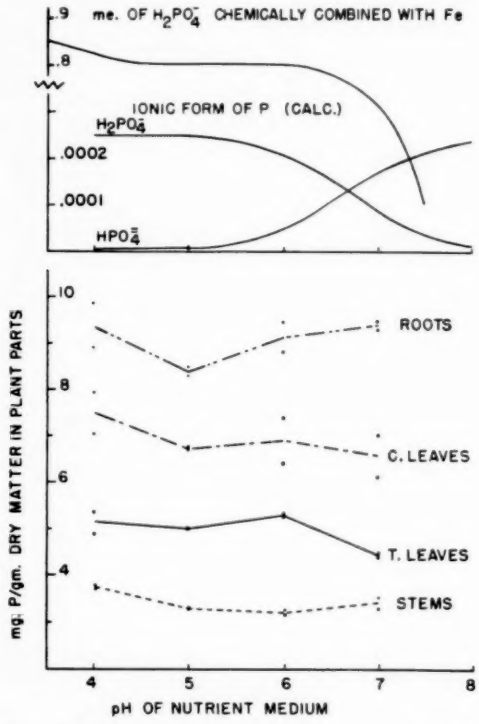

FIG. 1. Above: The amount of $H_2PO_4^-$ which is chemically combined with iron at various pH levels according to Swenson, Cole, and Sieling (reproduced by permission of the copyright holders) and the ionic form of phosphorus (calculated) expressed in molar concentrations at various pH levels. Below: The concentration of phosphorus in various parts of four groups of bean plants, each group grown in a similar nutrient solution (containing iron) but maintained at a different pH level. Results for each pH level are from two separate pans of six plants each. The curves represent the average value at each pH. C. Leaves, cordate or unifoliate leaves. T. Leaves, trifoliate leaves.

results are expressed graphically in figure 1. Several forms of iron have been employed with little difference in the amount and distribution of iron in the aerial parts of the plant. Ferric nitrate was used in this study.

The results indicate that there exists a characteristic uptake of phosphorus for each pH level of the nutrient medium. Total uptake is highest

from a medium of pH 4.0 with a minimum occurring at or near pH 5.0. A second maximum occurs at or near pH 6.0 with a general decrease at higher pH. The behavior of individual parts is interesting, but it should be clearly stated that the root concentrations include both absorbed and adsorbed phosphorus since we have not found a successful means of distinguishing between them. Our root values are, therefore, without great significance as to absorbed phosphorus. Stem concentrations decrease with decreasing acidity to pH 6.0, but they show an increase between pH 6.0 and 7.0. Leaf concentrations decrease markedly at pH 6.0 to 7.0. This indicates that movement of phosphorus from stems and petioles to leaf blades is impaired at pH 7.0. The resultant accumulation of phosphorus in stems and petioles at pH 7.0 constitutes a medium rich in phosphorus through which other ions being transported to the leaf blades must pass. Under conditions of high pH (7.0) and with accumulation of phosphorus, some difficulty in the successful passage of iron ions to the blades has been found (**10**).

As a result of this and other studies, a pH of 6.0 has been adopted as a suitable pH at which to grow this variety of bean while attempting to study the effect of iron upon the uptake of phosphorus. At this acidity and with the nutrient solutions employed, very little drift in pH occurs and there are no unusual effects of pH upon the internal distribution of phosphorus between plant parts.

Phosphorus uptake as a function of nutrient phosphorus concentration

We have found it possible to grow beans at a wide range of phosphorus levels. The lower and upper limits may be defined respectively as that concentration at which phosphorus deficiency occurs, and as that concentration at which chlorosis develops. In general, phosphorus deficiency symptoms are slow to develop because of the extreme mobility of phosphorus within the plant (**3**) and it is possible for metabolically active tissues to continue their growth at the expense of phosphorus in other parts of the plant. The phosphorus requirement of beans is not high. Levels as low as 0.00005 M supplied at the rate of one liter per plant per four days (less than 0.4 mg. P plant/day) have proved sufficient for plants having several trifoliate leaves. At the upper extreme, chlorosis becomes a problem in bean plants growing in a solution in which the phosphorus concentration is 0.005 M. Our experiments on phosphorus uptake were designed to: (a) demonstrate the tolerable levels of phosphorus both in the presence and absence of nutrient iron; (b) furnish information on the nature of the relationship between nutrient phosphorus and tissue phosphorus; and (c) furnish a basis for the selection of proper phosphorus levels to be employed in further experiments dealing with uptake and metabolism of phosphorus.

Experimental plants were grown at four levels of phosphorus, 0.00000, 0.00005, 0.0005, and 0.005 molar. At each of these phosphorus levels, two iron levels, 0.00000 and 0.000018 molar (1 p.p.m.), were used. The plants

received half-strength nutrient solution on the first day, and on the fourth and eighth days, the solutions were replaced with fresh full-strength nutrient. The pH of all nutrient solutions was maintained at or near 6.0. The plants were harvested when the first indications of chlorosis were clearly visible in those plants receiving 0.005 M phosphorus with no supplementary iron (10 days). At the time of harvest, the leaves of the plants grown with no supplementary phosphorus were a dark bluish-green which is typical of phosphorus deficiency in this species. The results are shown graphically in figure 2.

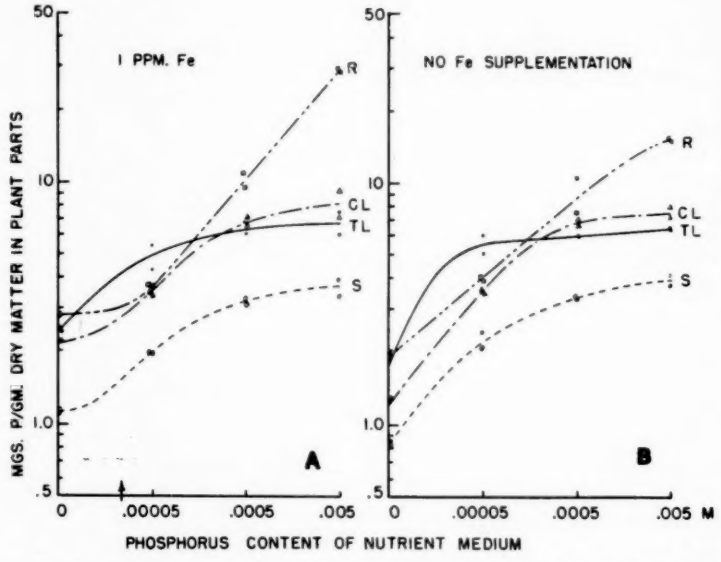

FIG. 2. A. The concentration of phosphorus in various parts of bean plants grown in nutrient solutions containing different amounts of phosphorus and 1 p.p.m. Fe. Results are from two separate pans of six plants each. The iron concentration (1 p.p.m.) would be equimolar with phosphorus at the position of the arrow on the abscissa. B. The concentration of phosphorus in various parts of bean plants grown in nutrient solutions containing different amounts of phosphorus, but with no iron supplementation. Results are from two separate pans of six plants each. R, roots; S, stems; CL, cordate leaves; TL, trifoliate leaves.

PLANTS GROWN WITH 1 P.P.M. IRON.—A ranking of plant parts with regard to their phosphorus concentration showed roots to be highest, with trifoliate and cordate leaves somewhat lower, and stems lowest of all. The concentration of phosphorus in the root was particularly high at the high levels of nutrient phosphorus, but the total amount included absorbed as well as adsorbed phosphorus, with the latter apparently contributing an appreciable amount. This is the characteristic distribution of phosphorus

between the various parts of the bean plant. Other elements, of course, assume their own characteristic distribution. No two are strictly alike.

As the concentration of phosphorus in the nutrient solution was increased from the lowest level upwards, all plant parts showed increased concentrations of phosphorus but the relation was not linear. Initially, the rate of increase was relatively low, particularly for root concentrations and to a lesser extent for stem and cordate leaf concentrations. Rapid gains were made only after the phosphorus concentration of the nutrient medium exceeded the iron concentration. The point of equivalence (equimolar concentrations) between iron and phosphorus in the nutrient medium is indicated by an arrow on the abscissa of figure 2 A.

The trifoliate leaves showed an initial rapid rise with the first increase in nutrient phosphorus, but the rate of rise was not as rapid as it was with plants receiving no supplementary iron. The accumulating power of these actively metabolizing leaves is very striking. The young trifoliate leaves, together with the meristematic tissues of the stem tip, were able to acquire and maintain higher concentrations of phosphorus from nutrient solutions of low phosphorus content (0.00005 M) than tissues of the root, stem, or cordate leaves. With further increases in nutrient phosphorus, tissue concentrations began to level off and lesser gains were made at the higher nutrient ranges.

The principal difference between the plants grown with and without iron in the nutrient medium, was in the phosphorus concentration of the respective parts of the plants grown at the zero level of nutrient phosphorus. Those plants which made their growth in the presence of an ample supply of iron had a distinctly higher phosphorus concentration than those receiving no supplementary iron. Of course, all growth in this case (zero P in fig. 2 A and B) was made by utilization of phosphorus which was present in the seed as no other source was available. The time allotted for growth (12 days) was sufficient to insure the utmost dilution of phosphorus which was possible under the conditions of growth. The failure of the plants supplied with iron to affect as great a dilution of seed phosphorus indicates a less efficient or thorough utilization of that phosphorus. The most logical interpretation of this observation is that at least a portion of the iron taken into the plant, or held on the root surfaces, was precipitated with a portion of the seed phosphorus rendering it unavailable for metabolic use. As a result, the tissues were relatively rich in total phosphorus at the time that growth ceased from a lack of metabolically usable phosphorus.

At the higher levels of nutrient phosphorus, the plants grown with and without supplementary iron were essentially similar in the phosphorus concentration of their tissues. Evidently, the amount of phosphorus held by iron is quite insignificant in comparison to the total phosphorus present when an ample supply is furnished to the roots.

PLANTS GROWN WITHOUT IRON SUPPLEMENTATION.—In those experiments where iron was intentionally omitted from the nutrient medium, and with

particular reference to the plants grown at the zero level of nutrient phosphorus, the concentration of phosphorus in the tissues of all plant parts is considerably lower than is the case where iron was present in the nutrient medium. Furthermore, the initial gain in phosphorus concentration was more rapid for all plant parts as the phosphorus content of the nutrient medium was increased. The initial flattened part of the curves as seen in figure 2 A is absent. The phosphorus concentration of the trifoliate leaves built up particularly rapidly indicating a completely unobstructed movement of phosphorus to these rapidly metabolizing parts. With further increases in nutrient phosphorus beyond 0.00005 M, only small gains were made. Beyond this point, the metabolic use of phosphorus by the trifoliate leaves, kept the incoming supply diluted to a value of approximately 6 mg. P/gm. of dry matter. Apparently this concentration within leaf tissue was adequate for normal metabolic functions.

The phosphorus content of roots, cordate leaves, and stems increased rather linearly with increases in nutrient phosphorus. At a nutrient value of 0.0005 M, a nutrient concentration 10 times as high as was necessary for maintenance of adequate trifoliate leaf concentrations, the phosphorus concentration of cordate leaves tended to level off. Stems and roots were still slowly increasing in phosphorus content and continued to do so even up to a nutrient concentraton of 0.005 M phosphorus. Thus it is evident that cordate leaves, stems, and roots progressively accumulate available phosphorus in excess of the values characteristic of these organs when the rapidly growing leaves of the upper stem are adequately supplied. It is this additional accumulation of phosphorus by roots, stems and cordate leaves, which appears in excess of the minimum requirements for the growth of the plant as a whole, that is responsible for disturbances in the metabolic use of certain other ions as these must enter and pass through tissues so rich in phosphorus as to cause precipitation reactions and immobilization (**5**). Iron is so frequently immobilized in this manner that we have adopted the term, phosphorus-induced-chlorosis, to designate this type of disturbance.

Experiments with tracer phosphorus.—In the above experiments the total uptake of phosphorus which had occurred during the experimental period was measured. In the experiment to be reported, the absorption and translocation of an aliquot of marked phosphorus was measured after a pretreatment period during which the different groups of plants were grown at phosphorus and iron levels indicated in figure 3. Plants were given half-strength solution at pH 6.0 during the first four days then full-strength solution for four days. This treatment was followed by a second four-day period in full-strength solution in which the added phosphorus contained P^{32} at a known P^{32}/P^{31} ratio. The plants were then analyzed for total phosphorus and marked phosphorus, the marked phosphorus being that phosphorus absorbed during the last four day period. One of the values of the tracer method lies in the fact that this fraction can be quantitatively measured even in the presence of the fraction which was absorbed previ-

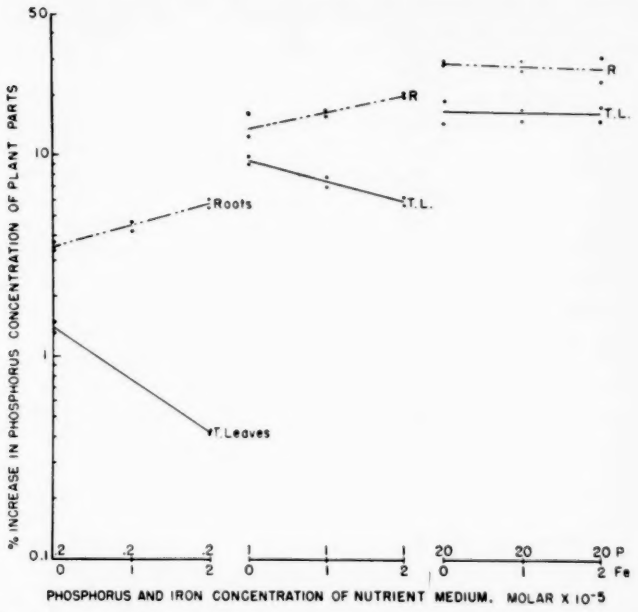

FIG. 3. The percentage increase in the phosphorus concentration of parts of bean plants as indicated by the accumulation of radioactive tracer phosphorus during a four-day interval prior to harvest. The various groups of plants were grown throughout (12 days) in nutrient solutions containing the phosphorus and iron concentrations indicated on the base lines. Results are from two separate pans of six plants each. R, roots; TL, trifoliate leaves.

ously. A measurement of the marked phosphorus present in each organ, as well as the total phosphorus, allowed the calculation of the percentage increase in phosphorus during the experimental period. The increase was calculated as follows:

$$\% \text{ increase in P} = \frac{\text{marked P} \times 100}{\text{total P} - \text{marked P}}.$$

The results are shown graphically in figure 3. The percentage increase in phosphorus reflects the amount of flow of currently acquired phosphorus into a given tissue. A high value in a selected tissue results if there has been an unimpaired flow of phosphorus to that particular area. A low value results when absorption is limited by the amount of phosphorus present, or by an impairment in the flow to the tissue.

By comparing plants grown at three different phosphorus levels, it is evident that the percentage gain in phosphorus is dependent first of all upon the amount of phosphorus present in the nutrient medium. Those plants growing at the highest nutrient levels acquire the most phosphorus. For

plants growing at the same phosphorus levels the determining factor in the acquisition of phosphorus is the amount of iron present. The roots gain phosphorus (adsorbed plus absorbed) in direct relation to the iron concentration of the nutrient medium, while the trifoliate leaves gain phosphorus in inverse relation to the iron concentration of the nutrient medium. This effect is greatest at the low nutrient phosphorus levels and becomes almost indiscernible at the high phosphorus levels. Iron, therefore, exerts a relatively greater influence in solutions of low phosphorus content. The effect is to cause a precipitation of phosphorus upon the roots which then obstructs the flow of phosphorus to the aerial parts. It will be seen that where there occurred ten or more atoms of phosphorus to each one of iron, the effect of iron upon the uptake of phosphorus into the trifoliate leaves was relatively insignificant. The explanation perhaps lies in the fact that the preponderance of phosphorus over iron is responsible for a rapid precipitation of the iron and a subsequent suppression of its occurrence in a soluble form in the nutrient medium. Plants are then growing in an essentially iron-free solution wherein ample phosphorus is present.

When iron is present in superabundance over phosphorus, the occurrence of ionic phosphorus in solution is suppressed by the formation of a ferric

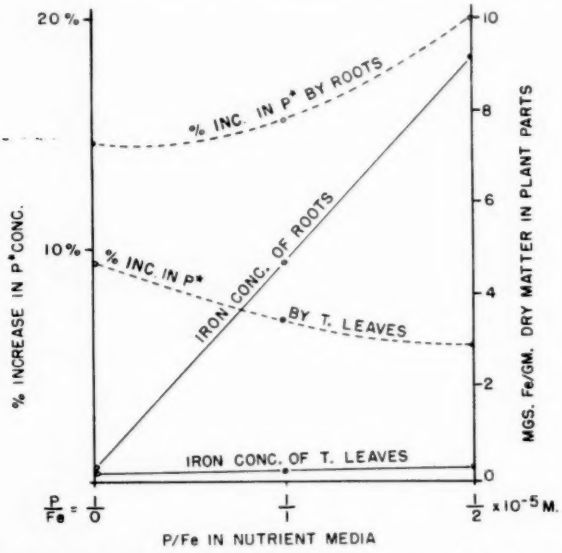

FIG. 4. The percentage increase in phosphorus, as indicated by the accumulation of radioactive tracer phosphorus during the four-day interval prior to harvest, and the corresponding iron concentrations at the time of harvest of parts of bean plants grown for 12 days in nutrient solutions containing various ratios of phosphorus and iron. Results are those of the central curves in figure 3 together with iron values as determined by John Rediske.

phosphate complex. The excess iron forms hydroxides which also contribute toward its precipitation. Iron, then, influences the uptake of phosphorus into the aerial parts of the plants first, by removing some phosphorus by precipitation, and second, by the formation of a precipitate upon the absorbing surfaces which impairs the rapid entrance and passage of phosphorus across the cortex of the root. Therefore, when precipitated phosphorus and iron accumulates upon the roots, the passage of phosphorus to the aerial parts is decreased.

The data from the 0.00001 M phosphorus level of figure 3 are presented in figure 4 together with the iron values for the roots and trifoliate leaves. This figure furnishes additional convincing proof that the interpretation of results on the basis of precipitation reactions is valid. The percentage gain in phosphorus by roots is greatest from solutions of high iron concentration. Likewise the amount of iron associated with the roots is also highest in solutions of high iron content. However, as the iron concentration and the percentage gain in phosphorus by roots increase, the gain in phosphorus by leaves is correspondingly decreased.

The nature of the precipitate which was present on the root surfaces has not been precisely determined, but the precipitate which formed within the nutrient solutions alone at pH values of 6 or lower consisted of approximately stoichiometric proportions of iron and phosphorus. At pH values above 6, some calcium was present in the precipitate and on a quantitative basis calcium plus iron was equal to phosphorus.

Swanson *et al.* (**13**) have shown that the following reaction takes place when ferric chloride is titrated with sodium hydroxide in the presence of an excess of phosphate:

$$Fe(H_2O)^{+++}_6 + 2\,OH^- + H_2PO_4^- \rightarrow Fe(H_2O)_3(OH)_2H_2PO_4 + 3\,H_2O.$$

The $H_2PO_4^-$ occupies only one of the coordination positions of the hexahydrated iron ions. It is assumed from the work of the above authors and from our own studies, that under the conditions of the experiments herein reported, *i.e.*, in nutrient solutions, the formation of the basic iron phosphate is possible and may take place to the extent of full participation of available phosphate ions up to the equivalence point. That phosphate which is present in superabundance over iron does not react and, except for some slight adsorption, remains available to the plant.

On the basis of this and other investigations, we now expect to find that a number of nutritional disturbances (other than chlorosis) will be explainable on the grounds of an immobilization of essential nutrients in precipitation and absorption reactions in the conductive tissues of the plant. It is known that certain soils are constituted, in part, of elements whose deficiency symptoms are often manifest in the plants which grow in them. The explanation frequently given is that the elements in question are tied up in the soil in a manner which renders them unavailable to the plants. From the data presented herein, it is shown that a more detailed explanation in-

volves precipitation reactions in and on the surface of the roots as well as in the conductive tissues, particularly near the distal ends, which renders some elements unavailable for metabolic use. Those elements which form insoluble precipitates with phosphorus are to be particularly suspected as participating in such a mechanism.

Summary

Absorption and translocation of phosphorus by bean plants was unimpaired at pH 4.0. A minimum in phosphorus accumulation occurs at or near pH 5.0 which reflects the slight solubility of basic ferric phosphate at or near this pH. It is conceivable that this minimum also reflects the minimal absorption encountered at the isoelectric points of certain cellular constituents active in phosphorus absorption. As the pH of the nutrient solution was raised from 6.0 to 7.0, the concentration of phosphorus in the stems and petioles increased, whereas the concentration in the leaf blades decreased. This observation indicates a lodging of incoming phosphorus in the stems and petioles at high pH values, creating tissues rich in phosphorus through which ions being transported to the leaf blade must pass.

As the phosphorus concentration of the nutrient medium is increased from low to higher values, the tissue concentrations also increase, but at different rates. The general effect is a rapid rise, followed by lesser increases until a leveling off occurs. The phosphorus concentrations of the nutrient solutions at which the different plant organs level off in phosphorus concentrations are as follows: trifoliate leaves, 0.00005 M; cordate leaves, 0.0005 M; stems, 0.005 M. Root concentrations begin leveling off at 0.005 M.

A concentration of approximately 6 mg. of P/gm. of dry matter in trifoliate leaves is attained from solutions at 0.00005 M P and this value is sufficient for continued growth of leaves. The corresponding combined stem and petiole concentration is approximately 2 mg. P/gm. dry matter when the leaves are adequately supplied. The stems and petioles will, however, build up to 4 mg. P/gm. dry matter as more phosphorus is made available. Cordate leaves correspondingly rise from 3 to 7 mg. P/gm. dry matter. It is this additional accumulation of phosphorus, beyond the concentration which is adequate for leaf growth and stem extension, which causes disturbances in the metabolic use of other ions, particularly iron. The passage of such ions through tissues rich in phosphorus is interfered with and much of the iron is precipitated along the conductive tissue. The principal effect of high phosphorus concentrations on the development of chlorosis is explainable on this basis.

In a nutrient solution containing both phosphorus and iron in nutrient quantities, a precipitate will form which will reduce the amount of available ions of both elements. The precipitate of iron and phosphorus forms both upon the container and upon the plant roots. The effect of the precipitation is twofold. First, it removes some of the materials from solution reducing

the effective concentration. Second, the presence of the precipitate upon absorbing surfaces furnishes a barrier to the rapid entrance of either species of ion, *i.e.*, phosphate and iron. Under certain conditions, precipitation reactions may also occur in the conductive tissues of the stem and leaf and so constitute a block in the transfer of certain ions from the conductive tissue to the leaf mesophyll and growing points.

The P^{32} herein employed was obtained from the Isotopes Division, Atomic Energy Commission, Oak Ridge, Tennessee.

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THE MECHANISM OF THE TETRAZOLIUM REACTION IN CORN EMBRYOS[1]

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(WITH TWO FIGURES)

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The reduction of tetrazolium salts to insoluble, colored formazans by living tissues was pointed out by KUHN and JERCHEL in 1941 (**18**). The reaction has since been widely used as an indicator of viability or metabolic activity with a variety of tissues and tissue extracts from animals (**6, 19, 28, 31**), microörganisms (**5, 11, 13, 22**), and higher plants (**9, 12, 27, 32**), especially seeds (**8, 10, 20, 25**). The 2,3,5-triphenyltetrazolium salts (hereafter called tetrazolium) have been used most commonly although several other derivatives have been introduced recently (**1, 2, 28**).

The biological reduction of tetrazolium has generally been attributed to enzymatic action. This interpretation was first suggested by KUHN and JERCHEL (**18**) on the basis of the low oxidation-reduction potential (–0.080 volts) and the lack of reduction by dead tissues or by ascorbic acid, sulfhydryl compounds, or sugars at physiological pH values. MATSON *et al.* (**24**) first reported that a dehydrogenase preparation (the glucose-6-phosphate system) would catalyze reduction, and several others (**19, 22, 28, 31**) have used the reaction as a dehydrogenase indicator.

Because of the increasing use of the tetrazolium test in the measurement of seed viability a more thorough study of the mechanism of the reaction in seed tissues was undertaken in 1949. This paper reports the determination of optimum conditions and the kinetics of tetrazolium reduction by malic dehydrogenase, the role of diaphorase, and the relative activities of several diphosphopyridine-nucleotide-linked dehydrogenases in corn embryos. Since this work was completed JENSEN *et al.* (**16**) have published further evidence that several pyridine-nucleotide-linked dehydrogenases would reduce tetrazolium, and BRODIE and GOTS (**7**) have shown that diaphorase may participate in the reaction.

Materials and methods

All enzyme preparations were made from seed of open pollinated WF9 × 38–11 hybrid corn. The seeds were soaked at 30° C for 18 to 20 hours with the embryo side down and half immersed in water. At this stage of germination there was visible radicle growth but seldom any emergence. Excised embryos were ground in cold 0.02 M phosphate buffer (pH 8) in a Potter-Elvehjem homogenizer to give a 10% suspension on a fresh weight basis. Cellular debris was removed by centrifuging cold for seven to eight

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minutes at about 1000 times gravity and the supernatant, which contained all the enzyme activity, was stored in an ice bath. There was no appreciable loss of activity for several hours and high levels of activity remained even after several days in the refrigerator.

Tetrazolium reduction was measured at 30° C in 15 ml. graduated centrifuge tubes containing 3 ml. of reaction mixture including buffer, substrate, diphosphopyridine-nucleotide (DPN), tetrazolium, diaphorase, and enzyme. Optimum conditions and concentrations are described in the following section. The reaction was stopped after 2 to 10 minutes by the addition of 6 ml. of dioxane which precipitated the enzyme and dissolved the formazan (2,3,5-triphenylformazan). The latter was then extracted by shaking with 1.5 ml. of xylene and centrifuging to give about 4.6 ml. of nonaqueous layer. The concentration of formazan was measured by the optical

TABLE I

FACTORS AFFECTING THE REDUCTION OF TETRAZOLIUM BY THE MALIC DEHYDROGENASE SYSTEM. R REPRESENTS RELATIVE RATES AS PER CENT. OF THAT UNDER OPTIMUM CONDITIONS (R = 100). OPTIMUM CONDITIONS WERE USED FOR OTHER COMPONENTS IN EACH CASE. AMOUNTS OF DIAPHORASE ARE GIVEN IN ML. OF THE STOCK PREPARATION IN REACTION MIXTURE.

Malate		DPN		Diaphorase		Glutamate		Tetrazolium		pH		Oxalacetate	
M	R	Mg.	R	ml.	R	M	R	M	R	pH	R	M	R
0	0–3	0	0–3	0	3	0*	40	0.008	14	8	5	0	100
0.017	35	0.5	20	0.05	8	0**	25	0.02	100	8.9	25	0.001	20
0.033	60	1.0	55	0.20	60	0.25	100	0.04	75–100	9.5	100		
0.050	85	1.5	100	0.60	100	0.50	100			9.9	24		
0.067	100	2.0	100	1.20	100					10.4	9		

* Glycine buffer.
** Ammonia buffer.

density at 490 mμ in 1 cm. cuvettes in a Beckman spectrophotometer. During extraction and measurement the solution was protected from strong light.

Methylene blue reduction was measured by the time for complete decolorization at 30° C in Thunberg tubes using 3 ml. of a reaction mixture containing 0.033 M phosphate (pH 8), 1 mg. DPN, 1.7×10^{-5} M methylene blue, 0.6 ml. of diaphorase preparation, and varying concentrations of substrate and enzyme.

The reduction of tetrazolium by chemically reduced DPN was carried out in the following reaction mixture: 0.25 micromole of reduced DPN, prepared by HOGEBOOM's technique (**14**) and standardized spectrophotometrically, 12 micromoles of tetrazolium, and 0.6 ml. of diaphorase preparation, all in 5.7 ml. of carbonate-bicarbonate buffer (pH 9.7). After a 10 minute incubation at 30° C, the formazan was extracted by a dioxane-xylene mixture and measured spectrophotometrically.

The diaphorase preparation was made from pig heart by a modification of STRAUB's method (**30**). The alkaline phosphate extraction was made in a Waring Blendor, and the supernatant, following 43° C incubation, was 0.8 saturated with ammonium sulphate in the cold. The precipitate, after being redissolved in water, was dialyzed free of ammonia; and the opalescent yellow solution was made slightly alkaline with Na_2HPO_4 and stored frozen. This preparation contained a significant amount of malic dehydrogenase activity which was destroyed by heating at 60° C for 10 minutes.

Other special materials used were the following: milk flavoprotein, prepared according to BALL (**3**); DPN (40%), 2,3,5-triphenyltetrazolium chloride, hexose-diphosphate, and hypoxanthine, Schwartz Laboratories; L-malic acid, L-glutamic acid, sodium α-glycerophosphate, Eastman; β-hydroxybutyrate, Fisher; oxalacetic acid, Organic Specialties.

Results

PROPERTIES OF THE MALIC DEHYDROGENASE-TETRAZOLIUM REDUCING SYSTEM

The enzyme preparation alone, dialyzed or undialyzed, caused very little reduction of tetrazolium. With the addition of any of several dehydrogenase substrates plus DPN, there was some increase in rate of reduction but maximum rates required the addition of a flavoprotein carrier, diaphorase. Since the malic system was especially active, it was chosen for detailed analysis of tetrazolium reduction by a DPN-linked dehydrogenase. Oxalacetate, product of the reaction, strongly inhibited the enzyme and had to be removed to maintain the rate. Cyanide, which has been used by others (**21**), increased the rate markedly at pH 8 but, at higher pH values, cyanide reduced tetrazolium and was unusable. Glutamic acid, acting presumably by transamination (**23, 26**), increased the rate several-fold and served both to bind oxalacetate and as a buffer. The inhibitive effect of added oxalacetate is shown in table I. The optimum pH of the system with glutamate was found to be about 9.5, and at pH 12 to 13 non-enzymatic reduction appeared. Other buffer salts, including borate, ammonia, and glycine, used with or without glutamate had variable effects on reaction rates but in no case gave a higher rate than gutamate alone. Phosphate generally increased the rates at pH 8 and occasionally at 9.5, but the results were not consistent and appeared to vary with the enzyme preparation. The effects of varying concentrations of malate, DPN, diaphorase, glutamate, and tetrazolium are shown in table I. The optimum levels indicated were used in all subsequent work. The low rate without malate showed that there was no significant glutamic dehydrogenase activity in either the diaphorase or the enzyme preparation at that level. Negligible rates without enzyme or with heated enzyme also showed the absence of malic dehydrogenase in the diaphorase preparation. Addition of nicotinamide (**21**) was not found to affect the rates or to reduce the DPN requirement.

Under the optimum conditions described, however, the reaction rates were not constant. Typical rate curves are shown in figure 1. In most

cases (curve 1) the rate increased during the first four or five minutes and then remained nearly constant. In fact, the first part of the reaction gave an approximately logarithmic increase in formazan concentration with time. In some cases (curve 2), however, no constant rate was reached during the 10 minute reaction period.

Since it appeared that the rate of tetrazolium reduction increased as the formazan concentration built up, at least to a certain level, the effect of adding formazan at the start of the reaction was investigated. Formazan was prepared both by enzymatic action and by dithionite reduction. In the latter case the suspension was stabilized with gelatin and washed with

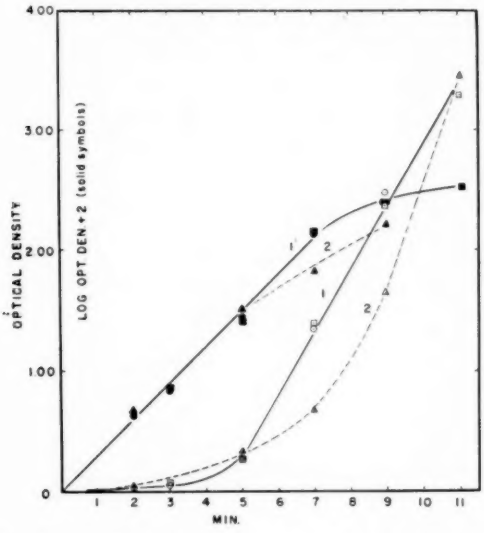

FIG. 1. The change in reaction rate with time. Optimum conditions were used. Curves 1 and 2 represent the types of rate curves observed. Outline symbols are for optical density, solid symbols for log of optical density.

oxygen to remove excess reducing material. Both type of formazan preparations were found to increase tetrazolium reduction under appropriate conditions. There was no evidence of formazan destruction in any of the reaction systems. Typical results are given in table II. Tetrazolium alone was not reduced by the formazan preparations nor was the complete enzymatic system affected by adding a dithionite gelatin blank (prepared without tetrazolium). Heating the enzyme, omitting malate, DPN, or diaphorase, or adding oxalacetate, all prevented extra tetrazolium reduction by added formazan, as well as blocking enzymatic reduction. Apparently the formazan effect involved the dehydrogenase itself. The effect was somewhat erratic, however, varying in magnitude and failing altogether in a few cases

(Exp. no. 6–27b). The reason for this variability was not clear, but it appeared to depend on the level of enzyme activity and the extent of the reaction. If the enzyme activity was such that the formazan concentration built up very rapidly in a normal system, *i.e.* without added formazan, an added amount at the start under similar conditions had a negligible effect on the enzymatic reduction. There was also evidence that the age of the homogenate may have affected the magnitude of the formazan effect. Whatever the basis of this effect may be, it seems to be involved in the production of the unusual rate curves observed.

TABLE II

FACTORS CONTROLLING THE FORMAZAN EFFECT. THE COMPLETE SYSTEM (CS) CONTAINED MALATE (M), DPN, DIAPHORASE (D), GLUTAMATE, TETRAZOLIUM (T), AND ENZYME AT OPTIMUM CONCENTRATIONS GIVEN IN TABLE I. FORMAZAN (F) CONCENTRATION WAS MEASURED BY OPTICAL DENSITY (OD). EXTRA [F] = $OD_{(CS + F)} - [OD_{(CS)} + OD_{(F)}]$. AMOUNT OF ADDED FORMAZAN IS INDICATED BY THE OD OF REACTION MIXTURE CONSISTING OF F ALONE.

Exp. no.	Reaction mixture	Time	[F]	Extra [F]
		min.		
4–13	F	20	1.31	....
	F + T	20	1.30	....
4–17	CS + F	10	2.96	0.89
	CS	10	0.76	....
	F	10	1.31	....
	CS − M	10	1.26	0
	CS − DPN	10	1.29	0
	CS − D	10	1.29	0
	CS + 0.001 M oxalacetate	10	1.40	0
4–19	CS + F	15	2.90	1.27
	CS	15	0.56	....
	F	15	1.07	....
6–27a	CS + F	15	1.10	0.43
	CS	15	0.24	....
	F	15	0.43	....
6–27b	CS + F	15	0.89	0
	CS	15	0.36	....
	F	15	0.58	....

Furthermore, reaction rates were not found to be directly proportional to enzyme concentration. Figure 2 shows the types of behavior observed ranging from curve 1 where increasing enzyme concentration gave a nearly linear increase in rate to curve 3 where a logarithmic increase resulted. The intermediate type, curve 2, was most commonly observed. The only conditions under which fair proportionality was achieved were with very low enzyme activities which gave formazan concentrations having optical densities below 0.2. These results again seem to indicate an accelerating effect of high formazan concentrations. With the present system no conditions have

been found which make tetrazolium reduction suitable for accurate dehydrogenase assay.

Tetrazolium reduction by chemically-reduced DPN

The role of diaphorase in DPN-linked tetrazolium reduction also can be demonstrated in the non-enzymatic reaction between dithionite-reduced DPN and tetrazolium. As shown in table III, no significant amount of formazan was produced unless diaphorase was added. The amount of reduced DPN was determined spectrophotometrically. Under the conditions used the reaction was apparently either slow or incomplete, but the catalytic action of diaphorase was clearly indicated.

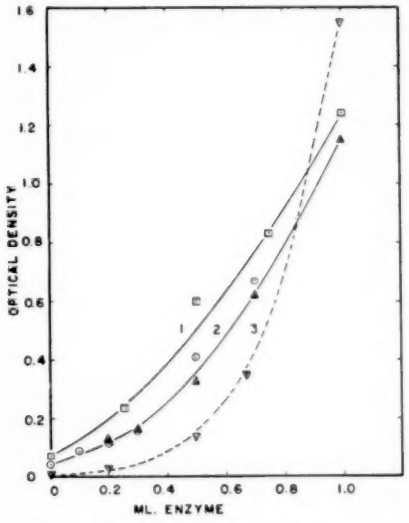

Fig. 2. Reaction rate as a function of enzyme concentration. Optimum conditions were used with a five minute reaction time.

The diphosphopyridine-nucleotide-linked dehydrogenases of the corn embryo

In addition to malate, four other substrates caused tetrazolium reduction in the standard DPN-diaphorase-homogenate system. Results are included in table IV. Alcohol dehydrogenase was the only one approaching the activity of the malate system. Both glutamic and β-hydroxybutyric dehydrogenases were very much less active. The reduction with hexosediphosphate was probably due to triosephosphate dehydrogenase after splitting of the hexose by aldolase. Some of the other substrates, especially glycerophosphate, gave slight reduction after much longer reaction times but no measurable amount of formazan during the standard 10 minute pe-

TABLE III

TETRAZOLIUM REDUCTION BY CHEMICALLY REDUCED DPN.

DPN · H_2	Diaphorase	Tetrazolium ml. 4%	Optical density 490 mμ, 3.6 ml.	Formazan
μM	ml.			μM
0.24	0.6	0.1	0.106	0.023
0.24	0	0.1	0.009	
0.24	0.6 (5 min. 100° C)	0.1	0.013	
0	0.6	0.1	0.006	

riod. The minimum reaction measurable under these conditions was equivalent to about 0.003 micromole of substrate oxidized.

The above substrates also were tested for methylene blue reduction using the same conditions with the Thunberg technique. Though rates by the two methods are not strictly comparable, the data in table IV do show that the embryo homogenate had a similar relative activity toward the various substrates in each case. Methylene blue reduction, however, was observed with several substrates which did not yield a measurable amount of formazan. Since complete methylene blue reduction was equivalent to 0.05 micromole of substrate oxidized and only one fifth as much enzyme was used in these cases, it was apparent that methylene blue was a more effec-

TABLE IV

COMPARISON OF TETRAZOLIUM AND METHYLENE BLUE REDUCTION BY VARIOUS SUBSTRATES IN THE CORN EMBRYO HOMOGENATE-DPN-DIAPHORASE SYSTEM, L-MALATE AT pH 9.5, ALL OTHERS AT pH 8.0.

Substrate	Methylene blue		Tetrazolium	
	Enzyme conc. (mg. fresh wt. per 3 ml.)	Time for complete reduction	Enzyme conc. (mg. fresh wt. per 3 ml.)	Optical density (10 min.)
		sec.		
L-Malate (0.05 M)	0.25	150	2.5	1.00
Alcohol (0.5 M)	0.25	240	2.5	0.100
β-Hydroxybutyrate (0.04 M)	10	100	50	0.083
Hexose-diphosphate (0.02 M)	10	180	50	0.072
L-Glutamate (0.05 M)	10	195	50	0.037
α-Glycerophosphate (0.03 M)	10	390	50	0
Citrate (0.1 M)	10	390	50	0
Succinate (0.1 M)	10	340	50	0
Glycine (0.05 M)	10	300	50	0
Hypoxanthine (0.01 M)	10	640	50	0
D-glucose (0.1 M)	10	600	50	0
Formate (0.1 M)	10	>600	50	0
D,L-Lactate (0.1 M)	10	>600	50	0
Hypoxanthine	0.1*	100	0.6*	1.375

*Ml. of milk flavoprotein.

tive hydrogen acceptor than tetrazolium. The results with malate and alcohol lead to the same conclusion.

Since reactions with citrate, succinate, glycine, and hypoxanthine required the addition of DPN, they were probably not catalyzed by the isocitric dehydrogenase-aconitase system or by succinic, amino acid or xanthine oxidases. These substrates must have been further metabolized to some DPN-linked dehydrogenase substrates (except, perhaps, in the case of citrate since there is evidence with some tissues (**15**) of conversion of DPN to TPN (triphosphopyridine nucleotide) which would allow isocitric dehydrogenase action and there is a recent report of a DPN-linked isocitric dehydrogenase (**17**)). Finally, no evidence for formic or lactic dehydrogenase activity was found by either method.

In addition to the DPN-linked dehydrogenases of corn embryo a milk flavoprotein containing xanthine oxidase was found to reduce tetrazolium (table IV). This observation and the diaphorase requirement of the DPN-linked systems indicate that flavoproteins are a characteristic part of the tetrazolium reducing mechanism.

Discussion

This work has shown that DPN-linked dehydrogenases coupled through diaphorase actively reduce tetrazolium. Substrates showed a wide range of activity from malate to glutamate and, with the exception of lactate, were the same as those giving a positive test with the corn embryo preparation of Jensen *et al.* (**16**). The latter workers found reduction without added diaphorase using a saturated ammonium sulphate precipitate from an aqueous extract of acetone-dried embryos. However, no quantitative measurements were made and there is no indication that their conditions were optimum for tetrazolium reduction.

Optimum conditions for the malic dehydrogenase of corn embryo homogenates with tetrazolium as hydrogen acceptor were found generally similar to those reported for other tissues and acceptors. Optimum malate concentration agreed with that of Berger and Avery (**4**) with Avena coleoptile and thionine and with that of Potter (**26**) with rat liver and the cytochrome system. The optimum DPN concentration was about half that found by Potter. The optimum pH was similar to that with Avena and with the dissociated kidney and liver preparations of Huennekens and Green (**15**), who used 2,6-dichlorophenolindophenol, except that the optimum range was much narrower. The corn embryo-malate system, like the dissociated cyclophorase preparation, showed a much smaller response to oxalacetate-binding agents at pH 9.5 than at 8 but even at the higher pH, glutamate increased rates considerably.

The incomplete reduction of tetrazolium by dithionite-reduced DPN which was observed may be explained by the recent report of Brodie and Gots (**7**) that the reaction is complete only under anaerobic conditions and that the optimum pH is lower than that used here.

The marked diaphorase stimulation of the corn embryo-tetrazolium system was also true of the Avena coleoptile-thionine system. Malic dehydrogenase in homogenates (**26**) or cyclophorase (**15**) preparations from mammalian tissues, however, do not require added diaphorase, though purified preparations may require it (**21**). The difference between the plant and animal preparations may be merely in the amount or stability of the diaphorase or in the degree of association with the dehydrogenase. However, the nature of the hydrogen acceptor may also be involved. The corn embryo-malic system showed a much smaller response to added diaphorase with an indophenol dye as acceptor (unpublished results), similar to that of Huennekens and Green, than with tetrazolium or methylene blue.

The malic dehydrogenase-tetrazolium reaction with the corn preparations was peculiar in the unusual changes in rate with time and enzyme concentration. The product of the reaction, triphenylformazan, in some way increased dehydrogenase reaction. Whether this action depends on the insolubility of the formazan or the low potential of the system, both of which distinguish it from other common hydrogen acceptors, or whether some other unknown property of the system is involved has not been determined. The same type of corn preparations with an indophenol acceptor, however, has shown no such behavior (unpublished results).

Another peculiarity in the tetrazolium method described is the high concentration of acceptor (0.02 M) required for maximum rates. Most of the other acceptors have been used at a level at 10^{-4} or 10^{-5} M. This fact and the lower rate of reduction of tetrazolium compared with methylene blue seem to indicate that the former is a less efficient acceptor. However, the tetrazolium method is aerobic and, although the formazan is not autoxidizable, it is possible that oxidases may compete as hydrogen acceptors. In fact, some evidence was found that cyanide, which poisons metal-containing oxidases as well as binding oxalacetate, would allow somewhat higher rates than glutamate at pH 8 though the point has not been adequately studied. With intact oat embryo tissue unpublished data have shown that tetrazolium reduction decreased with increase in oxygen atmospheres from 5 to 100%. The effect of oxygen tension on reduction rates in homogenates has not yet been fully investigated. Significant oxidase interference would be most likely with the higher homogenate concentrations and may explain the failure to observe tetrazolium reduction with some substrates active in the Thunberg technique. The failure of formazan production in the triosphosphate-DPN system of Brodie and Gots (**7**) also indicates that competing hydrogen acceptors may function under aerobic conditions.

It should be emphasized again that reduction resulting from the addition of a given substrate does not prove that dehydrogenase action is on that substance alone, especially if the activity is low. The behavior of succinate in the present work and that of Jensen *et al.* (**16**) illustrates the caution required in basing an assay procedure (**19**) on what may be a multiple enzyme system where there is no certainty as to which is the limiting step.

For further elucidation of the mechanism of tetrazolium reduction it will be desirable to extend the present results with purified enzyme systems.

At present a positive tetrazolium reaction in seed viability tests may be due to the action of pyridine-nucleotide-linked dehydrogenases or possibly to aerobic dehydrogenases such as xanthine oxidase. A weak or negative reaction may indicate (a) deterioration or lack of the dehydrogenases or diaphorase, (b) insufficient supply of substrates, or (c) competitive action of aerobic hydrogen acceptors.

Summary

Reduction of triphenyltetrazolium chloride by corn embryo tissues is catalyzed by diphosphopyridine-nucleotide-linked dehydrogenases, particularly the malic and alcohol systems, and is mediated by diaphorase. Optimum conditions and kinetics of the malic system were investigated, and a comparison of tetrazolium and methylene blue reduction with various substrates was made. The aerobic dehydrogenase, milk xanthine oxidase, also catalyzes the reaction.

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A KINETIC STUDY OF THE ABSORPTION OF ALKALI CATIONS BY BARLEY ROOTS

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(WITH EIGHT FIGURES)

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The absorption of inorganic salts by plant roots and other biological systems depends upon metabolic processes. When absorption proceeds against a concentration gradient, an expenditure of metabolically derived energy is obviously required. In some systems, such as barley roots, even absorption along a concentration gradient is negligible under conditions of arrested metabolism; and the concentration of the salt in the sap fails to reach the level of the outside solution (**12, 13, 17**).

The absorption process is characterized by a considerable degree of selectivity. The discrimination on the part of living cells between potassium and sodium is particularly striking. Most living cells accumulate potassium in preference to sodium, even when the external environment is rich in sodium and poor in potassium.

The view has come to the fore in recent years that both the role of metabolism and the specificity in the absorption process can best be accounted for by assuming that ion binding compounds are generated by metabolic processes. General reviews which make this point include those by USSING (**29**), ROBERTSON (**25**), and STEINBACH (**27**). OSTERHOUT (**22**) has succinctly stated some of the problems for whose solution such a concept would be useful. OSTERHOUT (**22**) and JACOBSON and OVERSTREET (**14**) have outlined several properties which the ion binding compounds must possess to account for characteristic features of the absorption process. These workers also stress the need for assuming that the complex is labile and breaks down again, releasing the ions.

COWIE *et al.* (**5**), ROBERTS *et al.* (**23**), CONWAY *et al.* (**4**), EDDY *et al.* (**7**), and EDDY and HINSHELWOOD (**8, 9**) have investigated the problem of ion binding in microörganisms, arriving at similar views. ROBERTS and his co-workers (**23, 24**) and EDDY and co-workers (**7, 8**) have related the binding of alkali cations to carbohydrate metabolism, and BOLTON and ROBERTS (**1**) have produced evidence that the hexose phosphates are responsible for the binding of potassium in *Escherichia coli*.

The hypothesis that the absorption of inorganic ions involves their combination with binding compounds forms the basis of the present study. Evidence will be presented for the existence of several distinct reactive sites involved in the binding of alkali cations by barley roots. It will be demonstrated that there are large differences in the affinity of these sites for various cations.

Methods

Roots of the Sacramento variety of barley were used as the experimental material. The method for obtaining barley roots with a high capacity for

ion accumulation was similar to that used by JACOBSON *et al.* (**15**). Briefly, 20 gm. of Sacramento barley seed were suspended in 40 ml. 10% H_2O_2 for 20 minutes, with occasional shaking. The H_2O_2 solution was decanted without rinsing, and the seeds were soaked for 24 hours in 1 liter of continuously aerated distilled water. The seeds were then rinsed several times with distilled water. Following this they were planted upon a layer of cheesecloth supported by means of a stainless steel screen over 4 liters of 10^{-4} M $CaSO_4$ in a 4-liter Pyrex beaker. There was a distance of approximately 2 cm. between the surface of the solution and the seeds. The corners of the square piece of cheesecloth dipped into the solution. A second stainless steel screen was placed on top of the seeds and covered with another piece of cheesecloth which also dipped into the solution.

The seedlings were grown in a dark chamber maintained at 24° C. The solution was continuously aerated by means of a sintered glass aerator. Forty-four hours after planting, the top screen and its layer of cheesecloth were removed. Three days after planting, the solution was renewed. At this time the roots were rinsed by removing the stainless steel screen from the beaker and dipping the roots in two portions of distilled water. The screen and plants were then transferred to another beaker containing 4 liters 10^{-4} M $CaSO_4$ and returned to the growth chamber. The roots were used five days after planting. The screen with the plants was removed from the beaker and the roots were rinsed by dipping them into two portions of distilled water. They were then excised just below the stainless steel screen and suspended in 4 liters of distilled water at 24° C. The roots were thoroughly mixed while in the beaker.

Rb^{86} obtained from the Isotopes Division of the Atomic Energy Commission, and K^{42} and Na^{24} prepared in the cyclotron of the Department of Terrestrial Magnetism, Carnegie Institution of Washington, Washington, D. C., were used as tracers. The Rb^{86} was received from Oak Ridge in the form of the carbonate. Excess HCl was added to a solution of the salt, and the solution evaporated to dryness. A 0.1 M stock solution of the chloride was prepared. It was often desirable to make isotopic dilutions of the stock solution by mixing aliquots of it with appropriate volumes of a 0.1 M solution of non-radioactive RbCl. Na^{24} and K^{42} were prepared essentially as described by COWIE *et al.* (**5**). The stock solutions of these ions also were 0.1 M solutions of the chlorides.

Determinations of radioactivity were made with a Geiger-Mueller counter of conventional design, using an end-window counting tube. Every sample was counted twice for a total count of 5000 each time. Self-absorption was negligible. Aliquots of the stock solution were counted at the same time, with the same geometry.

Roots were removed from the water in the beaker in which they were suspended, blotted on previously washed, dry cheesecloth to remove water adhering to the surface, and 1.00 gm. portions were weighed out on a torsion balance and transferred to Pyrex test tubes, 250 mm. × 22 mm., containing 50 ml. distilled water at 24° C. Each tube was equipped with an

aerating tube with inside diameter of 3 mm. The aerating tube was held in place by means of a wrap of non-absorbent cotton which also served to close the mouth of the test tube. For each sample, the entire operation of removing roots from the beaker, weighing and transferring them to the test tube took less than one minute. Just prior to the start of the experiment proper, the water in each tube was decanted and replaced by a volume of water, redistilled in an all-Pyrex still, equal to 50 ml. minus the volumes of salt solutions to be added to that tube. The tubes were transferred to a water bath maintained at 30° C and the aerators connected to a manifold. The requisite volumes of standard salt solutions were then pipetted into the tubes. When solutions of two salts were added to a tube, the salt of the radioactive cation whose absorption was to be determined was added first, followed immediately by the other salt. It was found that the order of addition did not significantly affect the results, provided that the interval between additions was brief.

The absorption period was three hours. The solutions were continuously aerated during that time. Under these conditions, the reduction in the concentration of the solution in the course of the experiment, due to absorption by the roots, was not pronounced enough to affect the results significantly, so that for the purpose of these experiments, the concentration of the solutions may be said to have been essentially constant during the absorption period. At the end of the absorption period, the solutions were decanted and the roots rinsed by filling the tubes with distilled water and decanting three times in succession. The root samples were transferred to one fourth oz. seamless-tinned-iron cups and ashed overnight at 450° C. A drop of Rocceal and approximately 1 ml. water were added and the samples dried. The Rocceal serves to reduce the surface tension and promote uniform distribution of the samples over the area of the cups. Radioactivity determinations were then made as described above.

It was found that replications in the same experiment gave values seldom differing by more than 5%. Replications were, therefore, considered unnecessary; and all of the absorption rates reported were obtained from single samples.

Experimental results

If two ions, A and B, are bound by the same binding group or site, each would be expected to interfere with the absorption of the other by competing with it for available sites. If, on the other hand, ions A and B are bound by different sites, A will not necessarily interfere with the absorption of B. Non-interference of ion A with the absorption of B must be interpreted as evidence that ion A is not bound by the same site as is ion B.

In experiments involving the absorption of Rb^* (mixture of the stable and radioactive isotopes) as affected by K, K was found to interfere with Rb absorption at all concentrations and ratios of the two ions tested. On the other hand, Na affected Rb absorption in a more complex pattern. (a) At Na concentrations above 10 m.e. l. Na interferes with Rb uptake (fig. 1,

left). (b) At Na concentrations of 10 m.e. /l. and below, Na fails to interfere if the concentration of Rb does not exceed 1 m.e. /l. (fig. 1). At higher Rb concentrations, Na even at low concentration has a depressing effect on Rb absorption. This effect becomes progressively less pronounced at higher Na concentrations, so that there is little, if any, difference in Rb uptake between 5 and 10 m.e. /l. Na (fig. 1).

These findings represent two distinct mechanisms of Na interference with the absorption of Rb. One of these (type a) does not become obvious until the Na concentration exceeds 10 m.e./l. The other (type b) produces its maximum effect at approximately 5 m.e./l. Na, doubling the Na concentration causing little or no further reduction in Rb uptake. This effect (type b) becomes less pronounced the lower the Rb concentration, and effectively disappears at a Rb concentration of 1 m.e. /l. These features of the effect

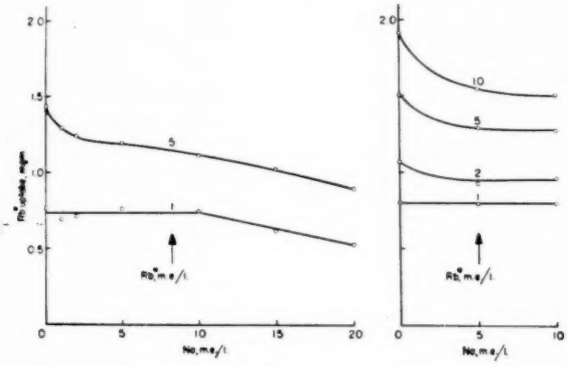

FIG. 1. Rb uptake as a function of the Rb and Na concentrations. Two experiments.

of Na at concentrations of 10 m.e./l. and below (type b) imply, according to the precepts outlined above, that the effect of Na at these concentrations does not involve direct competition of Na ions for the reactive groups or sites which bind Rb.

The apparent failure of Na at concentrations of 10 m.e./l. and less to compete for the Rb site is not due to non-absorption of Na at these concentrations. Figure 2 shows the results of an experiment on Na absorption as affected by Na concentration and aeration. The solutions of one set were aerated as usual, while N_2 gas was bubbled through the solutions of a parallel set. The gassing was started 30 minutes prior to the addition of the Na^*Cl. The aerated roots absorbed Na at an appreciable rate. The experiment also shows that Na absorption is not of a passive, non-metabolic nature, but is a function of aerobic metabolism like the absorption of K (**12, 13**) and Rb (**10**).

It has been assumed that the absorption of ions involves their combination with metabolically derived binding groups or sites. This combination must, however, be of a temporary nature, since the evidence indicates that the ions pass into free solution in the vacuole. The absorption process then is pictured as follows (**15, 22**): At the outer surface of a membrane

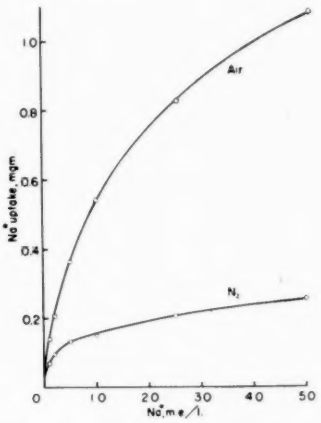

FIG. 2. Na uptake as a function of Na concentration and aeration.

which is impermeable to the free ions, the ions combine with metabolically produced binding compounds or carriers. They traverse the membrane in this form. Upon reaching the inner surface, the binding compound or carrier is chemically altered by metabolic processes so that the ions are set free.

These assumptions can be expressed in the following equations:

(1) $$R + M_{outside} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MR$$

(2) $$MR \underset{k_4}{\overset{k_3}{\rightleftharpoons}} R' + M_{inside}$$

where R and R′ represent different chemical states of the metabolically produced carrier, M the ion, MR the unstable carrier-ion complex, and k the rate constant for each reaction indicated. The over-all reaction is essentially irreversible. Under aerobic conditions, healthy roots do not lose significant amounts of potassium to distilled water (**13**).

Kinetic studies of enzyme reactions have led to the view (**11, 16, 20, 28**) that the substrate combines with the enzyme to form an unstable complex which then breaks down to yield free enzyme and the product or products of the reaction. These reactions are usually expressed in the following equations:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \tag{3}$$

$$ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \tag{4}$$

where E is the free enzyme, S the substrate, ES the enzyme-substrate complex, P the products of the reaction, and k the rate constants. The breakdown of ES is assumed to be practically irreversible, *i.e.* k_4 is negligible.

It will be seen that these views concerning the mechanism of ion absorption and enzyme activity are quite analogous, the carrier, R, corresponding to the enzyme, E, and the ion, M, to the substrate, S. The two processes differ in that the combination between enzyme and substrate produces a chemical change in the substrate molecule, whereas the combination of the carrier with the ion results in the transport of the ion from the medium to the interior of the cell. The carrier, R, rather than the substrate, undergoes a chemical change. The particular processes under study, however, do not affect the analysis of kinetic data (**16**).

It would be beyond the scope of the present paper to give a detailed treatment of the kinetics of enzyme reactions. This subject has been discussed by Ebersole *et al.* (**6**), Haldane (**11**), Lineweaver and Burk (**16**), Neurath and Schwert (**21**), and Wilson (**30**), among others. Only a brief outline will be given below, in order to indicate the manner in which this treatment will be applied to the problem of ion transport.

Straus and Goldstein (**28**) have shown that the above authors have based their treatments upon the assumption that the concentration of enzyme centers is constant and small compared with the concentration of substances with which they may combine. Likewise, in the present application of this treatment to the transport of ions, the assumption is implicit that in this case the number of binding sites is essentially constant during the experimental period, and their concentration small in comparison with that of substrate; *i.e.*, the system operates in Zone A of Straus and Goldstein.

Although the kinetic treatment and the terminology used here are those based on work with enzymes, it is stressed that it is not necessary to equate the ion binding carrier R with an enzyme, although this is indeed a likely interpretation (**1, 9, 18, 19**). While the type of kinetic analysis used here was first applied to enzymatic reactions, this type of analysis is valid whenever the mechanism of a reaction involves the general features embodied in equations 3 and 4, *i.e.*, the reaction of a substrate with a compound to form a labile complex which subsequently breaks down, some chemical or physical change occurring as the net result of this temporary combination.

Case 1. Reaction in absence of inhibitor.—From equations 3 and 4, a velocity equation can be derived:

$$v = \frac{V\,(S)}{K_m + (S)} \tag{5}$$

where v represents the observed velocity of the reaction at substrate concentrations (S), V the maximum velocity at which the enzyme is saturated with substrate, and K_s the Michaelis constant (**2**). LINEWEAVER and BURK (**16**) pointed out that equation 5 becomes linear in form upon taking the reciprocal of both sides:

$$\frac{1}{v} = \frac{K_s}{V\,(S)} + \frac{1}{V} \tag{6}$$

On plotting 1/v against 1/(S), a straight line results. The ordinate intercept is 1/V, and the slope K_s/V, thus giving a convenient method for determining both V and K_s.

All of the above considerations may be applied to the problem of ion binding. In this case, v and V will stand for the measured and maximum rate of absorption, respectively, (S) for the concentration of the substrate ion, M, in the solution, and K_s for the constant.

CASE 2. COMPETITIVE INHIBITION.—The kinetic analysis of enzyme reactions has been extended to include the effect of inhibiting substances. In competitive inhibition, the inhibitor combines with E at the same site or reactive group as the substrate so as to compete with the substrate for available sites. In this case, relative inhibition of the reaction will depend on the concentration of both the substrate and the inhibitor. The velocity equation in this case is:

$$\frac{1}{v} = \frac{1}{V}\left[K_s + \frac{K_s(I)}{K_i}\right]\frac{1}{(S)} + \frac{1}{V} \tag{7}$$

where v and V are the observed and maximum rates, respectively, K, the constant, and (I) and (S) the concentrations of the inhibitor and substrate, respectively. The symbol V in this and all other equations stands for the maximum rate in the absence of the inhibitor. It should not read V^* or V^1 (maximum rate in the presence of inhibitor) as has been repeatedly stated (**6, 21, 30**). Equation 7 differs from equation 6 in that there is an increase by the factor $[1 + (I)/K_i]$ in the slope of the line obtained upon plotting 1/v against 1/(S), with no change in intercept.

In applying these considerations to the problem of ion absorption, cations other than those whose absorption is under consideration will be looked upon as interfering ions, corresponding, to a first approximation, to the inhibitor in the case of enzyme reactions. In equation 7, (I) then will stand for the concentration of the interfering ion, v and V for the observed and maximum rates of absorption of the substrate ion, M_s, present in concentration (S), and K_s and K_i for the Michaelis constants of the complexes RM_s and RM_i, respectively, where R denotes the ion binding carrier and M_s and M_i the substrate and interfering ions.

CASE 3. NON-COMPETITIVE INHIBITION.—The inhibitor combines with

the enzyme irrespective of the presence of substrate, that is, it combines with both E and ES at a point different from the one to which the substrate attaches itself. The velocity equation is:

$$\frac{1}{v} = \left[1 + \frac{(I)}{K_i}\right]\left[\frac{1}{V} + \frac{K_s}{V} \cdot \frac{1}{(S)}\right] \tag{8}$$

in which the symbols have the meaning assigned them in equation 7. Equation 8 differs from 6 in that upon plotting 1/v against 1/(S), both slope and intercept are increased by the factor $[1 + (I)/K_i]$ so that the ratio slope/intercept remains constant. A means is thereby provided for distinguishing non-competitive from competitive inhibition, for in the latter the slope increases whereas the intercept does not, with the result that the ratio slope/intercept is increased. In terms of ion absorption, non-competitive inhibition represents the case where the interfering ion, M_i, is bound by a site or reactive group different from that by which the substrate ion, M_s, is bound, and equally so in the presence or absence of substrate ions.

Case 4. Un-competitive inhibition.—This case differs from non-competitive inhibition in that the inhibitor does not combine with both E and ES, but with ES only, at a site or reactive group different from that with which the substrate combines. The velocity equation is:

$$\frac{1}{v} = \left[1 + \frac{(I)}{K_i}\right]\frac{1}{V} + \frac{K_s}{V} \cdot \frac{1}{(S)} \tag{9}$$

The double reciprocal plot, 1/v against 1/(S), gives an unchanged slope, but the intercept is increased by the factor $[1 + (I)/K_i]$ (cf. equation 6). As a result, the ratio slope/intercept decreases. In the case of ion absorption, un-competitive inhibition denotes the interference of ion M_i with the absorption of ion M_s due to the combination of M_i with RM_s at a site different from that by which M_s is bound. In un-competitive inhibition, M_i does not combine with free R.

The following experiments will be interpreted in terms of the above analysis. Rb^+ will be the substrate ion, M_s, whose concentration was represented in the velocity equations by (S) and will be expressed in m.e./l. The other alkali cations will be looked upon as interfering ions, M_i, present in concentration (I). The observed velocity, v, of the reaction will be expressed as mg. Rb^+ absorbed/three hrs. After the first 30 minutes, the amount of Rb^+ absorbed is a linear function of time under the conditions of these experiments. The straight lines of the double reciprocal plots, 1/v against 1/(S), were calculated from the experimental points as linear regressions by the method of least squares (**26**). These calculations also yielded the values for the slopes and intercepts.

Figure 3 (left) shows the results of an experiment on the absorption of Rb as a function of the Rb concentration in the solution at three levels of K, plotted in the conventional manner. Figure 3 (right) represents a double

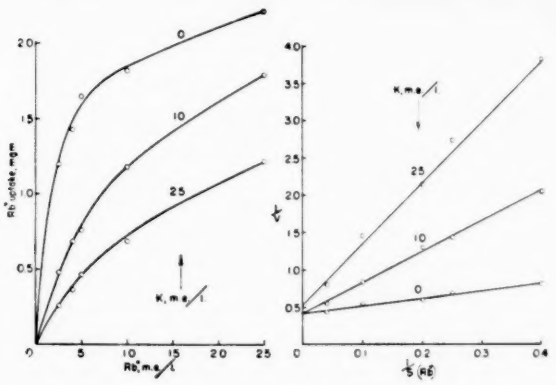

FIG. 3. Competitive interference of K with Rb uptake. Left: conventional plot. Right: a double reciprocal plot of the same data.

reciprocal plot, 1/v against 1/(S), of the same experiment. In the absence of K, a straight line is obtained with ordinate intercept $1/V = 0.423$, corresponding to a maximum absorption rate, V, at infinite Rb concentration, of 2.364 mg./three hrs.—the approximate value actually approached at high Rb concentrations (fig. 3, left). In the presence of K, the slope of the straight lines increased with little or no increase in the ordinate intercept and hence, an increased slope/intercept ratio—the characteristic features of the competitive type of interference.

Figure 4 shows the results of two experiments in which the Rb concentration was varied from 2 to 10 m.e./l. and K and Na were the interfering ions in the first (left) and second experiments, respectively. Comparison

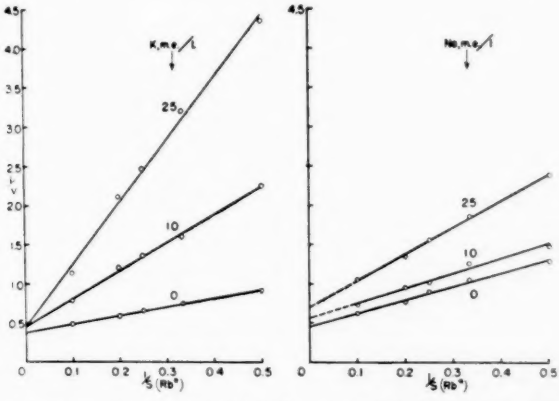

FIG. 4. Double reciprocal plots of interference of K (left) and Na with Rb uptake. Rb: 2 to 10 m.e./l. Two experiments.

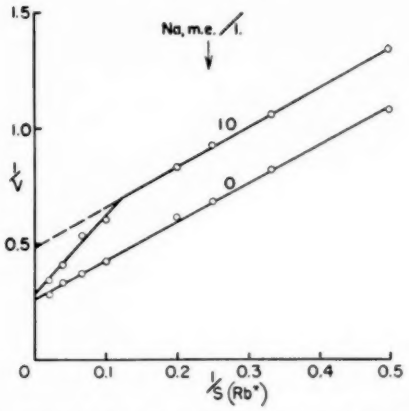

FIG. 5. Double reciprocal plot of interference of Na and Rb uptake. Rb: 2 to 50 m.e./l.

of the effects of the interfering ions at 10 m.e./l. shows that whereas K interferes in the manner characteristic of competitive inhibition (fig. 3), the line for Na = 10 m.e./l. is essentially parallel to the line obtained in the absence of interfering ion (un-competitive interference, case 4). At the higher Na concentration (25 m.e./l.), both the slope and intercept increase, the slope more so than the intercept, indicating a competitive type in addition to the un-competitive type of interference.

The interference by Na becomes partly competitive not only at Na concentrations higher than 10 m.e./l., but also when the Rb concentration exceeds that value. This is seen in figure 5, which shows the results of an experiment in which the Rb concentration was varied from 2 to 50 m.e./l.

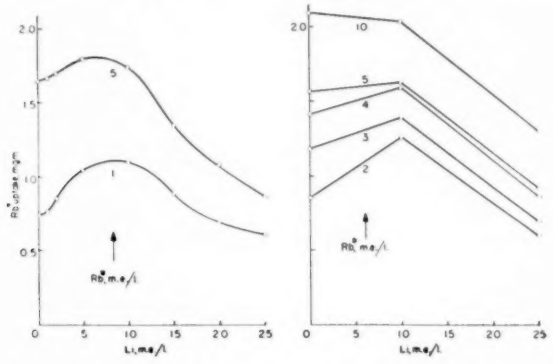

FIG. 6. Rb uptake as a function of Rb and Li concentrations. Two experiments.

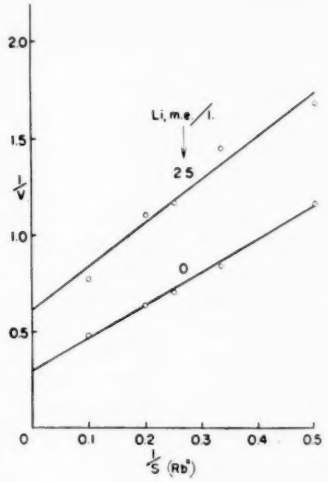

FIG. 7. Double reciprocal plot of interference of Li with Rb uptake. Rb: 2 to 10 m.e./l.

The effect of Li on Rb absorption under the conditions of these experiments is shown by the two experiments plotted in figure 6. The nature of the interference at high Li concentrations is not competitive as evidenced by the double reciprocal plot (fig. 7).

The interference of Cs with Rb absorption is competitive in nature (fig. 8). At the lower Rb concentrations, for Cs = 10 m.e./l., the values for

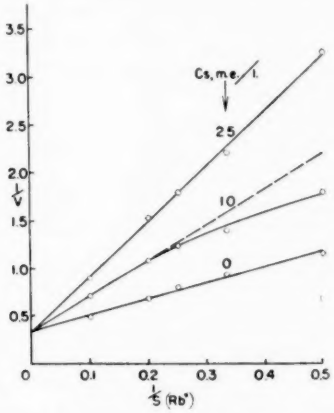

FIG. 8. Double reciprocal plot of competitive interference of Cs with Rb uptake. Rb: 2 to 10 m.e./l.

1/v are low, *i.e.*, the rate of Rb absorption is higher than would be expected on the basis of the extrapolation indicated in figure 8.

Discussion

The questions to which answers were sought in the present study may be summarized as follows: (a) Are the quantitative features of the absorption of alkali cations consistent with the view that the process involves binding in the form of a labile complex, as summarized in equations 1 and 2? (b) Is the absorption of alkali cations effected by one or by several types of sites? (c) If several sites are involved how do the various sites differ in their affinities for different cations? Specifically, are there sites which will differentiate between K and Na, preferentially binding the former?

In the absence of interfering ions, the double reciprocal plot, 1/v against 1/(S), consistently yields straight lines in experiments involving the absorption of Rb (figs. 3, 4, 5, 7, and 8) and K (unpublished). This result is expected on the basis of equation 6 which is based on the assumptions expressed in equations 1 and 2, and it is concluded that so far as these experiments are concerned, the first of the above questions is answered in the affirmative.

It is implicit in this conclusion, and in what follows, that the mechanism referred to is believed to be the only one that is of quantitative significance under the conditions of these experiments (very low salt roots, and physiological conditions of pH, temperature, and aerobiosis). There is no evidence in these and other experiments (**10**) to indicate that simple, nonmetabolic exchange not involving binding sites plays any but a minor role in determining the observed rates of Rb absorption.

Equation 6 shows that the straight lines obtained in the double reciprocal plots, 1/v against 1/(S), have a slope equal to K_s/V and intercept equal to 1/V, the ratio of these values yielding K_s. The values for K_s obtained (in the absence of interfering ions) in the experiments shown in figures 3, 4 (left and right), 5, 7, and 8 were, respectively, 2.44, 2.93, 3.74, 6.32, 5.82, and 4.86, all in m.e./l. The variability of the observed values of K_s in different experiments is perhaps not surprising in view of the fact that these experiments were not performed with purified substances *in vitro*, but with complex living tissues. Ebersole *et al.* (**6**), in studying nitrogen fixation by the free-living nitrogen-fixer *Azotobacter vinelandii*, found variations in the value of K_s of a comparable magnitude. In the present investigation there is suggestive evidence that the value of K_s is appreciably affected by the conditions obtaining in the period following the excision of the roots and prior to the start of the absorption period proper—an interval during which conditions were none too well controlled in these experiments. Furthermore, two different batches of Sacramento barley were used, and the seeds at the time of use varied in age, the work reported extending over a period of one year.

The second and third of the above questions will be considered jointly, for answers were sought through a kinetic analysis of the manner in which

the absorption of one ionic species is affected by the presence of another. The double reciprocal plots of Rb absorption in the presence of K (fig. 3, right, and 4, left) and Cs (fig. 8) exhibit the features of competitive interference; there is little or no increase in the ordinate intercept, accompanied by a pronounced rise in the slope. It is concluded that K, Rb, and Cs are bound by the same sites or reactive centers. This finding is not interpreted as an indication that these ions are equivalent in all metabolic reactions. The deviation of the line for Cs = 10 m.e./l. from the expected straight line relationship (fig. 8) will be discussed below.

Table I lists the experimental values, ordinate intercept and slope, and the constants calculated from these by using equations 6 and 7 for those experiments which involve Rb uptake in the presence of K and Cs (the competitive cases). The symbols have the meaning assigned them in the equations. Examination of the last two columns of table I reveals that the constants for Rb (K_s) and the interfering ions (K_i) are of the same order

TABLE I

COMPETITIVE INTERFERENCE WITH Rb ABSORPTION BY K AND Cs.

Figure	Interfering ion, m.e./l.	Ordinate intercept, $mg.^{-1}$	Slope, $mg.^{-1}/(m.e./l.)^{-1}$	Ks, m.e./l.	Ki, m.e./l.
5	K – 0	0.423	1.032	2.440	....
	10	0.423	4.170	....	3.289
	25	0.546	8.390	....	4.718
6 (left)	K – 0	0.374	1.095	2.928	....
	10	0.453	3.573	....	5.902
	25	0.450	7.966	....	4.955
11	Cs – 0	0.346	1.683	4.864	....
	10	0.345	3.720	....	8.216
	25	0.329	5.831	....	9.454

of magnitude. In no one experiment do the constants K_s and K_i differ by more than factor of 2. WILSON (**30**) has pointed out that the estimation of K_i is subject to relatively large errors unless the values used in calculating it are known with a rather high degree of accuracy.

The fact that the constants for Rb, K, and Cs are of the same order of magnitude indicates that the assignment of the terms "substrate" and "interfering" to any of these ions is arbitrary and a matter of convenience only. The ion whose uptake is being measured is called the substrate, and the other the interfering ion. As a matter of fact, both ions are competing substrates in the ion-binding reaction. The case of competing substrates has been dealt with by HALDANE (**11**).

Provided that the concentration of both Rb and Na does not exceed the value of 10 m.e./l., the double reciprocal plots yield straight lines which are essentially parallel to the base line obtained in the absence of Na (fig. 4, right, and fig. 5), so that ratio slope/intercept decreases. This result is characteristic of un-competitive interference described by EBERSOLE *et al.* (**6**). The interfering ion combines with RM_n, but not with free R, at a site

different from that to which M_s is attached. When either Na (fig. 4, right) or Rb (fig. 5) exceeds the value of 10 m.e./l., approximately, the type of interference changes in a manner which indicates a competitive component as well. With respect to the former case (Na > 10 m.e./l., fig. 4, right), this change is interpreted as indicating that while at low concentrations, Na is bound by sites other than those which bind Rb, at higher concentrations Na "spills over," as it were, onto the Rb sites. The latter case (Rb > 10 m.e./l., fig. 5) indicates that at least two types of sites are involved in the transport of Rb in the presence of Na. At low Rb concentrations, Rb is bound by sites which the un-competitively affected by Na, provided that the concentration of the latter does not exceed 10 m.e./l. (fig. 5, the parallel section of the curve Na = 10 m.e./l.). At higher Rb concentrations, its absorption involves sites which also bind Na (fig. 5, the inclined portion of the curve Na = 10 m.e./l.).

When the concentration of substrate ion (Rb in this case) is progressively lowered, the proportion of the complex MR will decrease and the proportion of free R increase. Since in un-competitive interference the interfering ions (Na) have an effect upon MR, but not upon free R, it follows that the effect of Na should progressively diminish as the Rb concentration, and hence the concentration of the complex, MR, approaches zero. Figure 1 shows that this is indeed the case. At Rb concentrations of 1 m.e./l. and less, the proportion of MR (the only form affected by Na) in the system is so low that the Na effect becomes negligible.

The difference in the behavior of K and Na is brought out in the two experiments shown in figure 4. These experiments were identical except that K was the interfering ion in one (left) and Na in the other. For K as the interfering ion, the ratio slope/intercept increased from 2.928 for K = 0 m.e./l. to 7.887 for K = 10 m.e./l., whereas for Na at the same concentrations, the slopes were almost identical and the ratio slope/intercept decreased from 3.738 to 3.433. Hence K shows the characteristics of competitive interference with respect to Rb, whereas Na at the same concentrations, accompanied by the same anion, produces the characteristics of un-competitive interference, indicating a different binding site.

Comparison of equation 9 with equation 6 shows that in un-competitive interference, there is no change in the slope of the double reciprocal plot, while the intercept increases by the factor $[1 + (I)/K_i]$. In table II are listed the experimental values, ordinate intercept and slope, and the constants calculated from these by using equations 6 and 9 for the experiments with Na as the interfering ion. Only those cases are listed which do not involve concentrations of either Na or Rb higher than 10 m.e./l., since under those conditions a competitive component is present in addition to the un-competitive interference. As in the competitive case the qualitative characteristics of the interference can be demonstrated consistently in successive experiments, but the magnitude of the constants, K_s, and especially K_i, varies rather widely indicating the presence of uncontrolled factors which vary from experiment to experiment.

The interference with Rb uptake by Li at high concentrations (fig. 6) is not competitive (fig. 7). Both slope and intercept increase, the slope by a smaller factor than the intercept, indicating a type of interference intermediate between the un-competitive and the non-competitive. In this case the interfering ion affects both MR and R, with a greater effect upon the former.

At low concentrations of both Rb and Li, a positive effect on Rb absorption was shown to outweigh the negative effect which predominates at high concentrations (fig. 6). Similarly, at low concentrations of Rb and Cs there seems to exist a positive effect on the rate of Rb absorption in addition to the competitive interference. As a result, the interference at low concentrations of Rb and Cs (fig. 8) is less (Rb uptake is higher) than would be expected on the basis of the interference at the high Rb concentrations. Another interpretation of the features exhibited by the line for Cs = 10 m.e./l. may be that at low concentrations of Rb and Cs the interference of Cs with Rb uptake is of the un-competitive type (fig. 5). This deviation from the expected straight line relationship occurs in experiments involving the effect of Cs on the absorption of K as well.

TABLE II

UN-COMPETITIVE INTERFERENCE WITH Rb ABSORPTION BY Na.

Figure	Na, m.e./l.	Intercept, $mg.^{-1}$	Slope $mg.^{-1}/(m.e./l.)^{-1}$	Ks, m.e./l.	Ki, m.e./l.
6 (right)	0	0.455	1.701	3.738	....
	10	0.556	1.909	....	45.050
7	0	0.262	1.656	6.321	....
	10	0.489	1.712	....	11.542

Briefly then, K, Rb, and Cs have been shown to be competitive with respect to one another, *i.e.*, they are bound by the same binding sites or reactive centers. In the presence of Na, two types of such sites can be distinguished. At low concentrations of substrate ions, the sites involved do not bind Na, but at higher substrate ion concentrations (> 10 m.e./l.) additional sites are brought into play which also bind Na. The specificity of the former type site for K, Rb, and Cs is not absolute as evidenced by the fact that Na at concentrations above 10 m.e./l. becomes competitive toward it. Li is not competitive toward this K-Rb-Cs site.

COLLANDER (3) studied cation absorption by 21 different species of higher plants which were grown for approximately two months in complete nutrient solutions containing several cations in equivalent amounts. He found that all plants studied absorb Rb and Cs with almost the same rapidity as K. He found a similar relation between Ca and Sr. No other cations studied showed such distinct mutual correlations in their absorption rates. He also found that K and Rb mutually interfered with each other's uptake somewhat as though they were identical ions or two isotopes of the same element.

It will be apparent that these are precisely the results which would be expected for ions which are bound by the same binding sites at essentially the same rates. The agreement between the two studies is particularly significant in view of the widely different techniques employed. Collander grew plants in complete nutrient solutions for approximately two months, whereas the present study involved excised roots and three-hour absorption periods. The fact that Collander's findings were drawn from experiments with some 20 species suggests that the concepts elaborated in the present study may also be found to be of rather general validity. The findings of the investigators of mineral metabolism in microörganisms and considerations of a comparative biochemical nature would point in the same direction.

Summary

Experiments are described concerning the absorption of alkali metal ions (primarily Rb) by excised barley roots. The main independent variables were the concentration of the ion whose absorption was under consideration (the substrate ion) and the concentration of another alkali cation present (the interfering ion). The anion was chloride throughout.

The results are interpreted by means of a kinetic analysis based on the hypothesis that the absorption process entails the formation and breakdown of an intermediate labile complex, MR, of the metal ion, M, with a metabolically produced binding compound or carrier, R. The analysis is formally identical with the kinetic treatment of enzyme reactions based on the combination of the substrate, S, with the enzyme, E, to form a labile complex, ES. In this analysis, the interfering ions assume the role of inhibitors or alternate substrates.

It was found that K and Cs interfere competitively with Rb absorption, and it is concluded that these three ions are bound by the same binding sites or reactive centers.

Except at high Rb or Na concentrations, Na does not interfere competitively with Rb absorption, that is, is not bound by the same sites. At low Rb concentrations, Na over a wide range of concentrations entirely fails to interfere with Rb absorption. Li is not competitive with regard to the K-Rb-Cs sites, *i.e.*, it is not bound by them. At relatively low concentrations of Rb and Li, Li increases the rate of absorption of Rb.

The findings are considered to be consistent with the hypothesis and indicate the existence of several distinct binding sites of which one group binds K, Rb, and Cs in preference to Na and Li.

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A COMPARATIVE STUDY OF GRADUAL AND ABRUPT CHANGES IN ROOT TEMPERATURE ON WATER ABSORPTION[1]

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(WITH SEVEN FIGURES)

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Introduction

The effects of low root temperature on various plant processes have long been known and extensively studied. It is generally recognized that one of its important effects is the reduction in water absorption of the plant; and the nearer the root temperature approaches the freezing point of water, the greater this effect will be manifested. The change in physical state, particularly the increase in viscosity with the decrease in temperature, of both water and protoplasm are undoubtedly two basic factors influencing water absorption at low root temperatures. The reduction in water absorption will affect other plant processes, directly or indirectly, to a greater or smaller degree depending on the extent of water shortage within the plant, and consequently may result in corresponding injury to the plant. Conditions favorable to high transpiration have been reported to bring about wilting and injury to the plant from desiccation when its roots are subjected to low temperature. However, different plant species appear to differ in their capacity to withstand the effects of low root temperature. Kramer (**7**) observed that species which grow in the winter and species native to cooler climates are less affected by low soil temperature than warm-weather species. The statement by Kramer (**7**), which will be discussed later, that plants cooled slowly wilted less severely than plants cooled rapidly, seems to be the only report in the literature on the comparative effects of rates of lowering root temperature. Under natural conditions, the change in temperature varies not only in respect to the level to which the temperature falls but also in the rate at which it occurs. Information regarding the effects of different rates of temperature decrease seems at the present to be limited, and it was the purpose of this investigation to compare the effect on water absorption of an abrupt decrease in root temperature with a gradual decrease in root temperature.

Materials and methods

In the present investigation three plant species were used, sunflower (*Helianthus annuus* L.), tomato (*Lycopersicum esculentum* Mill.) variety Rutger, and red kidney bean (*Phaseolus vulgaris* L.) variety Black Valentine. Seeds were allowed to germinate in flats of moist sand or on cloth netting stretched over the top of a glazed crock of tap water. Seedlings

[1] Papers from the Department of Botany and Plant Pathology, The Ohio State University, Columbus 10, Ohio, no. 545.

were then transferred to glazed crocks or a large shallow tray containing a complete culture solution. When they reached the size to be used in the experiment, uniform plants were selected and transferred to black painted one-quart mason jars containing 700 ml. of the complete culture solution, one plant to each jar. At all times the plants were provided with uniform moderate aeration.

Control of root temperature was made by immersing the jars in which the plants were growing in temperature-controlled water baths. The two baths used in the experiments were rectangular metal tanks, seven feet long, two feet wide, and 10 inches deep, provided with an inlet and an outlet for water which was maintained at a depth of five inches (the level of the solution in the jars). The bath in which temperature was maintained constant at 25° C had a heating system consisting of a flexible type immersion heater operated by a bimetal thermoregulator connected to a silent power relay and a cooling system of running water from the water supply line. The other bath in which temperature was gradually lowered to 5° C had a heating system similar to that of the constant temperature bath and a cooling system consisting of a thermoregulator-controlled circulating pump which pumped ice water from a reservoir tank into the bath. By these methods it was possible to maintain the temperature at any desired level within a range of ± 0.1° C. Stirrers were kept running all the time during the period of the experiment to maintain uniformity of temperature throughout the baths. Temperature gradients in the baths were almost non-existent, the variation in temperature between the two ends of the tanks being no greater than 0.5° C. The gradual change in root temperature was made by making a small change daily in the setting of the thermoregulator in one of the baths. A temperature record was made from the readings of thermometers immersed in the water near each end of the tanks.

The decrease in volume of culture solution in the containers in which the plants were growing was used as the measure of water absorption. The volume of solution in each jar was measured daily by transferring it to a graduated cylinder, and the solution was then filled up to its original volume of 700 ml. with distilled water previously cooled to the same temperature. The time required for the measurement of water absorption by all the plants in each experiment was about one and a half hours. Absorption was measured in the same order throughout each experiment in order that every plant would have completed its 24-hour absorption period at the time when its measurement was made. Possible sources of error inherent in the method were (a) in the loss of water through evaporation as the solution was continuously aerated and (b) in the wetting of the walls of the containers and the roots when the solution was transferred for measurement. A test conducted to determine the magnitude of error from these sources showed that it was not greater than 2 ml. per day. In order to obtain substantial values for the absolute amount of water absorption and also to minimize the effect of error in the measurement, the plants were used when they were well be-

yond the seedling stage and had fairly high rates of water absorption. Under conditions favorable to rapid transpiration the average amount of water absorbed per day per plant of the control sets was more than 100 ml. for bean and 200 ml. for tomato and sunflower.

Prior to the start of each experiment the plants were allowed to remain in the 25° C water bath for a period of not less than two weeks. Gradual and abrupt changes in root temperature were then made in the following manner. The temperature of one water bath was lowered 1.5 or 2.0° C each day until it had been gradually reduced from 25 to 5° C. This rate of change in root temperature was considered as the gradual change in the present study. Abrupt changes varied in magnitude from 5 to 20° C and were made by transferring plants from the 25° C bath to a bath cooled to the desired temperature. In all treatments involving an abrupt change, the change was made only on the first day, after which the root temperature of these plants was gradually lowered until the final temperature of 5° C was reached. The following temperature treatments were made:

Set	*Change in root temperature*
Control	Constant at 25° C
I	Gradual from 25 to 5° C
II	Abrupt from 25 to 20° C, gradual from 20 to 5° C
III	Abrupt from 25 to 15° C, gradual from 15 to 5° C
IV	Abrupt from 25 to 10° C, gradual from 10 to 5° C
V	Abrupt from 25 to 5° C

Results and discussion

In the present investigation the daily rates of water absorption of the sets under treatment are expressed as percentages of the rate of the control set at 25° C for the same 24-hour period. The absorption rate of each set treated was also corrected for its relative rate of absorption to that of the control when both sets were subjected to the same 25° C root temperature prior to the treatment. This correction factor was used throughout the period of the treatment on the assumption that there was no change in the basic absorptive capacity of the sets except that due to the treatment given. As all plants were exposed to the same conditions of light intensity, ambient temperature (other than root temperature) and vapor pressure of the atmosphere, the use of this correction factor will remove the differences due to the effect of the above factors on transpiration and in turn its effect upon absorption of water by the plants in the different sets. The method of expressing rate of absorption as stated above was also used by KRAMER (7).

The effect of gradual and abrupt changes in root temperature on water absorption of sunflower plants six weeks old is presented graphically in figure 1. During the period of this experiment the weather was mostly clear or occasionally partly cloudy, favorable to a high rate of transpiration. It will be noted that the gradual change in root temperature generally resulted in a somewhat higher rate of water absorption than abrupt changes. In all

cases of an abrupt change in root temperature the absorption rate on the first day when the treatment was initiated fell sharply to a level slightly below that of the gradual change, and thereafter followed the general trend of absorption of the gradual change. At root temperatures of 8° C and below, wilting was observed during the middle of the day from 11:00 A.M. to about 2:00 P.M. on all plants (root temperature abruptly changed from 25 to 15° C and 25 to 10° C respectively). The wilting began on the lower leaves and progressed to the higher leaves; it was not severe enough to cause drooping of the stems, and lasted for only a brief period of two or three hours. Plants with root temperature abruptly changed from 25 to 5° C wilted severely about one and a half hours after being transferred to 5°

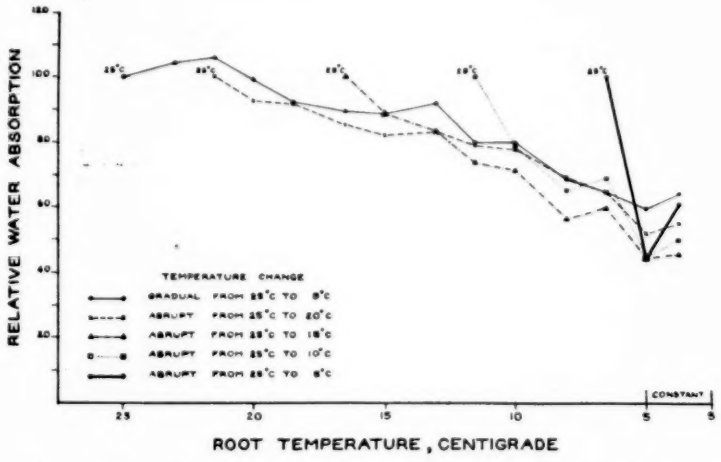

FIG. 1. Effects of gradual and abrupt changes in root temperature on relative water absorption per day of sunflower plants. The measurement of water absorption and change of root temperature were made at 8:00 A.M. The gradual temperature change from 25 to 5° C occurred over a period of 13 days. All sets were kept at 5° C for two days.

C, and remained in such condition throughout the day until about 6:00 P.M. when they began to regain turgidity slowly. Portions of some fully developed top leaves were desiccated. However, on the following day at the same root temperature, these plants appeared to wilt much less severely than on the first day, and the degree of wilting was about the same in sets III and IV. Plants in set I (root temperature gradually changed) and set II (root temperature abruptly changed from 25 to 20° C then gradually changed thereafter) did not show any sign of wilting throughout the range of root temperature, although their daily rates of absorption did not show very appreciable deviations from those of the wilting sets.

The results obtained with tomato plants eight weeks old are presented in figure 2. The weather conditions were favorable for high rates of tran-

spiration throughout the period of the experiment. The curves indicate an appreciable increase in the rate of absorption in the range of root temperature from 25 to 18° C. It is possible that this range of root temperature was the optimum range for water absorption in the particular variety of tomato used. Below 18° C the rate of absorption began to decrease with the decrease in root temperature. The rate of fall in water absorption was greater in tomato than in sunflower. In general the absorption rate of the plants receiving a gradual change in root temperature was greater than those of other groups. With the exception of the plants in set II which showed an increase in the rate of absorption when the root temperature was changed from 25 to 20° C, all plants in the other sets showed an abrupt drop

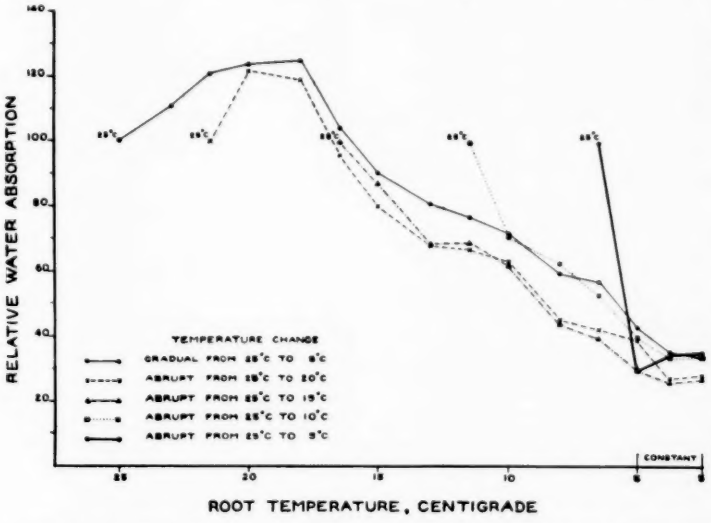

FIG. 2. Effects of gradual and abrupt changes in root temperature on relative water absorption per day of tomato plants. The gradual temperature change from 25 to 5° C occurred over a period of 13 days. All sets were at 5° C for three days.

in the absorption rate on the first day when their root temperatures were abruptly changed. The pattern of changes in the absorption rate of these groups were similar to those of sunflower plants. Wilting occurred only in the last set of plants which had the root temperature abruptly changed from 25 to 5° C. The wilting began about two hours after the root temperature was changed and was severe enough to cause drooping of all leaves and the top portions of the stems. The plants remained in the wilted condition throughout the rest of the day and slowly regained turgidity at night. Although the plants wilted very severely no injury from desiccation occurred. In the following days the plants in this group again wilted at about 11:00 A.M. but regained their turgidity sooner than in the first day (about 5:00 P.M.). The relative rate of absorption of the plants in this set showed

a slight increase from the drop of about 70% in the first day. When root temperature was lowered below 13° C guttation could not be observed from any plant so treated, but it occurred freely from the plants in the control set. Senescence of lower leaves occurred in all plants with their roots subjected to low temperature except those of the last set. The leaves gradually turned yellow and slowly dried out. All the leaves of the plants in the control set remained in good vegetative condition. About one third of the total number of fully developed leaves of each treated plant were senescent at the termination of the experiment. The beginning sign of senescence of some of the lower leaves of the last group was apparent from a gradual decrease in intensity of the green color of those leaves. It seemed that these plants

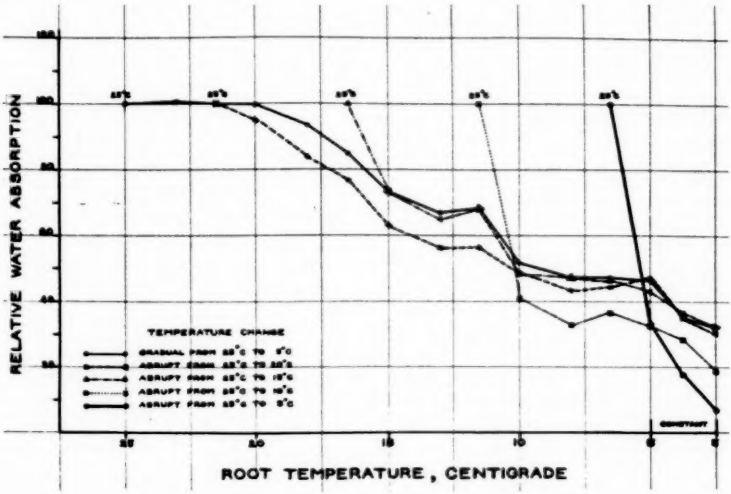

FIG. 3. Effects of gradual and abrupt changes in root temperature on relative water absorption per day of red kidney bean plants. The gradual temperature change from 25 to 5° C occurred over a period of 13 days. All sets were kept at 5° C for three days.

were not kept under the treatment long enough for the change to reach its more advanced stage.

Figure 3 shows the results of the experiment with red kidney plants six weeks old. The plants were exposed to the same atmospheric conditions as the tomato plants. Variation in root temperature within the range of 25 to 20° C caused very little change in the rate of absorption. When the root temperature decreased below 20° C, the rate of absorption began to decline more markedly. The rate of absorption of the plants receiving a gradual change in root temperature was generally higher than those receiving an abrupt change in root temperature. At 10° C the decrease in the absorption rate was approximately 50 to 60%, which was much greater than in

sunflower and tomato. Although the decrease in the absorption rate at root temperatures below 10° C was less conspicuous than above 10° C, the decrease progressed steadily even while the root temperature was maintained at 5° C. Wilting did not occur in the plants in sets I, II, and III throughout the entire course of the experiment. Plants in set IV wilted severely about two hours after the root temperature was changed abruptly from 25 to 10° C, and the wilting continued through the rest of the day until early at night when the plants gradually recovered. On the following day the injury due to desiccation of several whole leaflets or portions of some leaflets could be observed in all plants. The extent of the injury from desiccation was approximately 20% of the total foliage of each plant. The uninjured portion of the foliage showed slight wilting again on the following two days and remained turgid thereafter despite being kept at lower temperature; however, the rate of absorption continued to decline. Plants in set V which were transferred abruptly from 25 to 5° C were affected in the same way by the sudden drop of root temperature as those in set IV, but the wilting and injury from desiccation were much more severe than in the former set. Desiccation occurred in most of the fully developed leaves as well as in some of the young leaves and appeared to continue into the second day, and the injury damaged approximately 90% of the total foliage of the plants. The rate of absorption in this set continued to fall at a fast rate to 6.5% at the end of the third day when the root temperature was maintained at 5° C.

Several possible causes of reduction in water absorption at low root temperature have been given by KRAMER (**8**), and the more important ones are as follows: 1. Retardation of root elongation. Continual root growth is necessary to bring the absorbing regions of roots into contact with soil water in the undepleted portion of the soil. 2. Decrease in rate of movement of water from soil to roots. 3. Decreased permeability of cells. 4. Increased viscosity of protoplasm and of the colloidal gels in the cell walls. 5. Increased viscosity of water. 6. Decreased metabolic activity of the living tissues of the roots, which probably affects the permeability of protoplasmic membranes and the active-absorption mechanism.

The first two causes would not limit absorption in the present work inasmuch as the plants grew in solution, and thus the absorbing surfaces of the roots were constantly in contact with water. This point seems to be borne out by the work of ARNDT (**1**) who found that under conditions favorable to rapid transpiration, cotton plants wilted when the soil temperature was not lower than 17 to 20° C; but when growing in solution culture, wilting occurred at lower temperatures (10 to 17° C).

Two physical changes directly resulting from the effect of low temperature would be the increase in viscosity of water and of protoplasm. The specific viscosity of water is 0.4987 at 25° C, 0.5608 at 20° C, 0.6363 at 15° C, 0.7297 at 10° C, 0.8475 at 5° C, and 1.0000 at 0° C. It will be seen that the viscosity of water increases at an increasing rate as the temperature approaches its freezing point, and the viscosity increases at an increasing

rate from 10 to 0° C. The increase in viscosity results in a decrease in the diffusion pressure of water, and undoubtedly retards the rate of diffusion of water into root cells and through the cells of various tissues of the roots. The viscosity of protoplasm is also known to be greatly increased at low temperatures. KRAMER (**6**) cited the work of Weber and Hohenegger indicating that the viscosity of protoplasm in the root cells of Phaseolus seedlings was about four times as great at 2.0° and three times as great at 5.0° C as at 19 to 22° C. HEILBRUNN (**4**) showed that the protoplasmic viscosity of Amoeba increased greatly in the temperature range of 12 to 15° C and continued to increase but at a slower rate in the range of temperature below 12° C; the viscosity between 5 and 10° C changed only slightly and

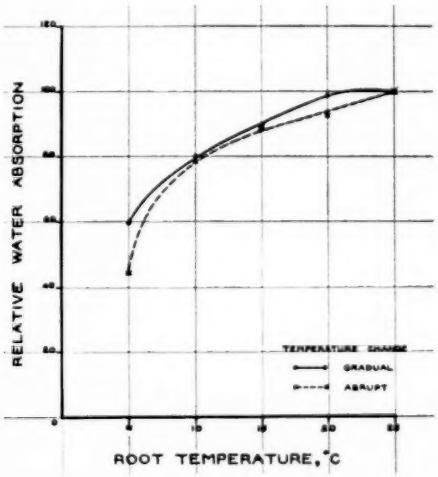

FIG. 4. A comparison of the effects of gradual and abrupt changes in root temperature on water absorption of sunflower plants. On the curve showing the effect of an abrupt change each point represents the rate of absorption of a different set of five plants in which the roots were abruptly cooled from 25° C to the specified temperature.

was about three times as great as at 25° C. The marked increase in viscosity of protoplasm at low temperature will certainly create some increase in resistance to the movement of water through the cells. KRAMER (**5**) found that the rate of absorption of sunflower plants in potometers at 6° C root temperature was increased two and one half times when the roots were excised, and the plants regained their turgidity very rapidly. He concluded that the root pressure mechanism was not the essential part in absorption and that the high resistance to water movement across the tissues into the xylem of the root was effective in slowing down the entrance of water. The same investigator (**6**) showed that the rate of water movement through dead sunflower roots attached to a vacuum pump also decreased with decreasing

temperature, but not to the same extent as in living roots, and stated that killing of the cells resulted in collapse of the protoplasts and disorganization of the strands of cytoplasm passing through the cell walls, thus lessening the resistance to water movement.

Although it is certain that the decrease in root temperature will affect the metabolic activity of the living tissues of the roots, which may in turn affect the rate of absorption, it is unlikely that the decreased metabolic activity will significantly influence the rate of absorption in rapidly transpiring plants. In the present investigation the plants with their root temperature gradually lowered were exposed to low temperature much longer than the plants with their root temperature abruptly changed, and yet the rates

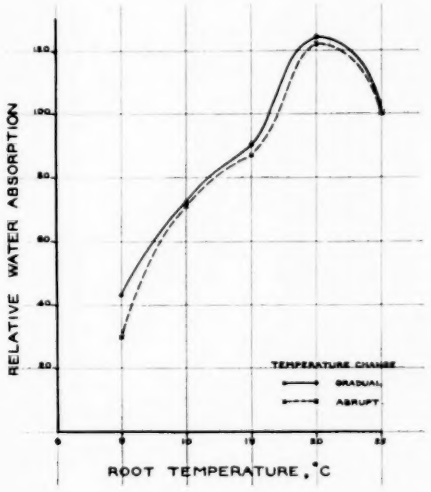

FIG. 5. A comparison of the effects of gradual and abrupt changes in root temperature on water absorption of tomato plants. On the curve showing the effect of an abrupt change each point represents the rate of absorption of a different set of four plants in which the roots were abruptly cooled from 25° C to the specified temperature.

of absorption fell to practically the same level. In general the results of the present investigation show that there was greater reduction in the rate of absorption at temperatures below 10° C in sunflower (fig. 4) and tomato (fig. 5) than at higher temperatures. This finding seems to agree with the changes in viscosity of water as discussed previously and shown in figure 7. In bean, however, the most rapid decrease in absorption was in the temperature range of 10 to 17° C corresponding closely to changes in viscosity of protoplasm as discussed above and diagrammed in figure 7. The curve representing absorption of water by bean plants seems to have the same general shape as would a curve drawn from figure 7 interrelating the effect of the viscosity of both water and protoplasm. It thus appears that the increase

in the viscosity of water and of the protoplasm of the root cells are two major factors causing the decrease in the rate of water absorption at low root temperature.

KRAMER (7) in an extensive study on the rate of absorption of water by various species of plants in cold soil found that serious reduction in water absorption was not limited to temperatures near freezing, but might occur in some species at temperatures between 10 to 20° C. He observed that absorption was reduced more in species which normally grow in warm soil, such as cotton and watermelon, than in species such as collards and northern pines which normally grow in cooler climates. He also stated that species differences with respect to the effect of low root temperature are probably

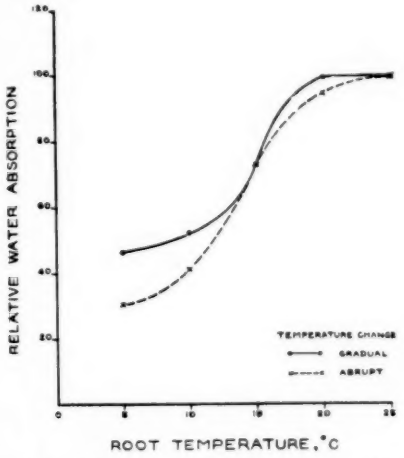

FIG. 6. A comparison of the effects of gradual and abrupt changes in root temperature on water absorption of red kidney bean plants. On the curve showing the effect of an abrupt change each point represents the rate of absorption of a different set of four plants in which the roots were abruptly cooled from 25° C to the specified temperature.

due to fundamental differences between species in the reaction of protoplasm to low temperature, possibly differences in the degree of change in viscosity and permeability of protoplasm. In this investigation the results also tend to show the presence of species differences in protoplasmic reaction to low temperature. It will be seen from figure 6 that the change in rate of water absorption by bean plants in relation to the change in root temperature seems to follow more closely the change in viscosity of protoplasm than the change in viscosity of water (fig. 7), indicating that in this species perhaps a more pronounced change in viscosity of protoplasm accompanied cooling. However, in sunflower (fig. 4) and tomato (fig. 5) the change in rate of water absorption appears to follow the change in viscos-

ity of water (fig. 7), indicating the probability that only a slight change in viscosity of protoplasm occurred in these species at low temperature and that certain protoplasmic changes might have occurred which may have reduced the viscosity changes in protoplasm as a limiting factor in water absorption. The change in rate of water absorption of bean appears to follow the general trend of changes in rate of transpiration of watermelon and cotton as reported by KRAMER (7), whereas that of sunflower and tomato tends to resemble the curve of the rate of transpiration of collards which can thrive in cold soil (7).

An abrupt decrease in root temperature generally resulted in an abrupt decrease in water absorption. Although the rate of absorption under the

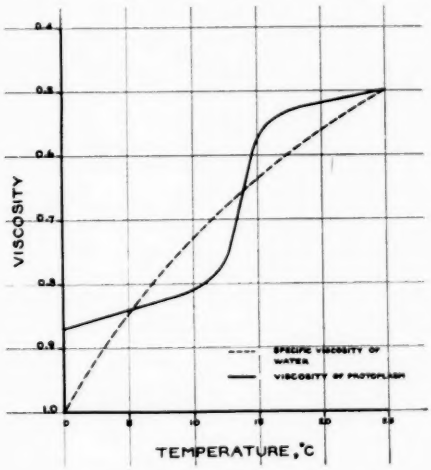

FIG. 7. Change in viscosity of water and protoplasm in relation to change in temperature. The curve for viscosity of protoplasm is based on the works of HEILBRUNN (4) and Weber and Hohenegger as cited by KRAMER (6).

gradual change of root temperature was generally higher than the rates under abrupt changes, the difference was not very great except when such abrupt change brought about some permanent injury to the plants as in the case of bean plants. KRAMER (7) observed that elm, privet and sunflower plants cooled to one or two degrees above freezing in four or five hours or even over night wilted severely, while plants cooled over a period of four or five days wilted only slightly, and the rapidly cooled plants also transpired less than plants which were cooled slowly to the same temperature. In the present investigation, none of the plants showed any sign of wilting when the roots were gradually cooled from 25 to 5° C, or when abruptly changed from 25 to 20° C and from 25 to 15° C then gradually cooled to 5° C. Bean

plants wilted severely when the temperature was changed abruptly from 25 to 10° C and from 25 to 5° C, while sunflower and tomato plants wilted severely when the temperature change was from 25 to 5° C; however, in the following days all the plants gradually recovered from the effect of such abrupt temperature changes, with the exception of the bean plants in set V (abrupt temperature change from 25 to 5° C) which were very severely injured by desiccation and did not appear to recover during the period of the experiment. BARNEY (**2**) also reported that the transpiration rate of loblolly pine seedlings was generally reduced when the soil temperature was suddenly decreased, and then the transpiration rate gradually increased toward a stable rate at that temperature. In the present work, it will be noted that the rate of absorption of the wilted plants was not much lower than that of the turgid plants, and it seems unlikely that the difference in water absorption at the same temperature between the two groups of plants was the cause of such greatly contrasting effects of the gradual and abrupt changes in root temperature. It is possible that transpiration rate may have had considerable influence in bringing about the contrasting results between the two groups of plants. The plants subjected to gradual change in root temperature showed a gradual decrease in rate of absorption. The decreased rate of water absorption would cause an increasing water deficit and a decrease in vapor pressure of water within the plants, which consequently would result in a decrease in the transpiration rate. The plants could remain at a low root temperature without apparent sign of wilting probably because the transpiration rate was continually decreasing as the root temperature was gradually lowered, and it did not therefore exceed the absorption rate enough to cause wilting. An abrupt decrease in root temperature might have resulted in an unbalanced condition between the rate of absorption and the rate of transpiration. The vapor pressure of water in the leaves, however, might not have changed as abruptly; and the plants would continue to transpire near the usual rate while water absorption was greatly retarded, thus wilting would have ensued. It might be expected that the high transpiration rate would not continue long, and would be greatly reduced as wilting advanced. The wilting of the plants was less severe on the following days, and this might be due to the gradual shifting of the transpiration rate toward an equilibrium with the reduced rate of water absorption. However, in the case of bean plants the loss of water from wilted leaves seemed to continue at such an appreciable rate that considerable damage from desiccation occurred to the foliage. TAGAWA (**11**) found that the stomates in bean plants opened more widely when the root temperature was abruptly lowered from 20 to 10° C, 5° C, and 0° C and remained widely opened while the leaves wilted; no injury from desiccation was reported probably because the experiment was of short duration. Continued opening of the stomates in the wilting leaves could cause further diminution in water content of leaf cells to the extent that extensive injury from desiccation occurred to the leaves.

Although lowering of root temperature will generally result in a decrease in the rate of water absorption, the effect seems to vary with the plant species. Some plant species appear to be able to withstand the effect of low root temperature better than others. CLEMENTS and MARTIN (**3**) found that the transpiration rate of sunflower plants varied very little with soil temperatures between 13 and 37° C, but fell rapidly as the temperature was decreased below 13° C. SCHROEDER (**10**) observed that cucumber plants might be severely injured by wilting when the root temperature dropped below 16° C. RALEIGH (**9**) found that muskmelon plants growing in solution culture wilted when the root temperature was lower than 18° C. The results of the present work also indicate that there are differences among the plant species used in the experiments, with respect to ability to withstand the effect of low root temperature. Bean plants appeared to be more susceptible to injury caused by abrupt decrease in root temperature than the other two species, and sunflower seemed to be less affected than tomato or bean. The rate of absorption of bean plants fell rather rapidly even at relatively high temperatures, while the absorption rate of sunflower plants began to drop rapidly only at relatively low temperatures.

Summary

The change in rate of water absorption under gradual and abrupt changes in root temperature within the range of 25 to 5° C was studied by measuring daily the decrease in volume of the culture solution in which sunflower, tomato and red kidney bean plants were growing. In general, the gradual decrease in temperature resulted in a gradual reduction in the absorption rate, and the abrupt changes in temperature resulted in a correspondingly abrupt decrease in the absorption rate to a value slightly below the absorption rate of the plants which had been gradually cooled to that same temperature.

The absorption rate of bean plants showed greater reduction in the temperature range of 10 to 17° C than at lower temperatures and appeared to follow the trend of reported changes in viscosity of protoplasm, indicating the probability that in this species the change in viscosity of the protoplasm is an important factor in controlling water absorption. The absorption rate of sunflower and tomato plants, however, showed greater reduction at temperatures below 10° C than at higher temperatures and tended to conform mainly with the reported changes in viscosity of water, indicating that certain protoplasmic changes may have occurred, which might have reduced the viscosity changes in protoplasm as a limiting factor in water absorption.

All plants with root temperature gradually decreased from 25 to 5° C showed no sign of wilting. Tomato and sunflower plants wilted severely on the day when the temperature was dropped abruptly from 25 to 5° C and gradually recovered on subsequent days with little or no permanent injury; but bean plants wilted severely and were greatly damaged from desiccation of the foliage when the temperature was abruptly dropped from 25 to 10° C

and from 25 to 5° C, and showed very slow recovery of the uninjured portion of the foliage.

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THE PERMEABILITY OF NON-STOMATE LEAF EPIDERMIS TO CARBON DIOXIDE

W. M. Dugger

(WITH FOUR FIGURES)

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Introduction

It has been generally assumed that the exchange of gases between the intercellular spaces of leaves and the external atmosphere occurs to an appreciable extent only when the stomates of the leaves are open. However, attempts to correlate the observed rate of CO_2 absorption either with the number of stomates per unit area or with stomate area have met with only limited success. Verduin (**16**) believed that the lack of correlation between diffusion rates and degree of stomate aperture was attributable to the interference of diffusion shells. Mitchell (**8**) has reported that stomates on leaves of Cineraria apparently were closed before wilting occurred, yet these plants in some instances absorbed 70% as much CO_2 as the control plants. In a study of the effect of soil moisture on photosynthesis of apple leaves, Schneider and Childers (**12**) found on several occasions high rates of photosynthesis when the stomates appeared to be completely closed. Verduin and Loomis (**15**) found that wilted maize plants absorbed less CO_2 than turgid plants. However, the decrease in CO_2 absorption was not proportional to the decrease in stomate size as measured by a porometer. The average absorption of CO_2 by wilted leaves was 37% of the controls.

Considerable variation in the absorption of CO_2 through the epidermal cells of leaves during photosynthesis was observed by Freeland (**4**). In all of his experiments, Freeland found that the rate of apparent photosynthesis was greater when CO_2 entered through the stomate epidermis than when it entered through the non-stomate epidermis; and it appears from Freeland's work that the amount of CO_2 entering through the epidermal cells is a function of the thickness of the cuticle. In plants with thin cuticle the amount of CO_2 that entered the non-stomate surface was approximately equal to the amount which diffused through the surface with stomates. In plants with thick cuticle little or no apparent photosynthesis could be detected when CO_2 contacted only the non-stomate epidermis.

Cutin is not an impervious barrier. Water loss is known to take place through the cutinized epidermal walls of leaves and stems. Furthermore, CO_2 is known to be soluble in cutin so it seems very possible that molecules of the gas may penetrate the cutinized wall of the epidermal cells and reach the internal cells of the leaf. Roberts *et al.* (**11**) used microchemical methods to show that the cutinized epidermal layer of McIntosh apple leaves contains a large amount of pectinaceous material. This material appears in the outer walls of epidermal cells as parallel and vertical layers, whereas

the cutinized layers are parallel. The presence of the pectinaceous layers in the epidermal cells could account for the entrance of water and water-soluble substances such as carbon dioxide.

Although there is considerable indirect evidence that the epidermal cells of plants are permeable to gaseous CO_2, no comprehensive study of this phenomenon has been undertaken. The purpose of this investigation was to determine directly whether cutinized epidermal walls of the leaves of several species of plants are permeable to carbon dioxide.

Materials and methods

Plants without stomates in the upper epidermis were selected for study. Preliminary experiments were conducted with Poinsettia, Coleus, Hydrangea, and Hedera. Coleus and Hydrangea were chosen for further study. The leaves of these plants are structurally suited for experimental work of

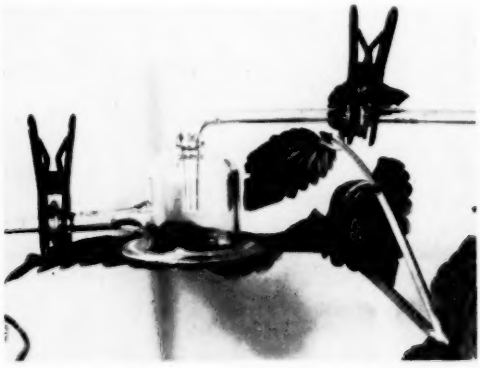

FIG. 1. A leaf cup sealed to the upper surface of a Coleus leaf.

this kind. The plants grow vigorously in the greenhouse and are easily maintained in the laboratory. The Coleus was of a variety containing a double factor for chlorophyll and the leaves were entirely green. The experimental plants were taken from the greenhouse on the day they were to be used and returned after the selected leaves had been exposed to carbon dioxide.

The apparatus for exposing the leaf to CO_2 consisted of a special leaf cup, figure 1, as part of the gas flow system previously described by the author (**2**). The leaf cup was sealed with liquid latex to the upper or lower surface of a leaf as described by WILSON (**17**). The latex coagulated and made a good seal within one hour. The seal was checked for leaks and the entire plant was placed in the hood with the ground glass joints of the cup connected to the gas flow system. Coleus and Hydrangea leaves were treated with 1, 5, and 10% carbon dioxide in air. Experiments were performed with the cup attached to the lower or to the upper surface of leaf.

Penetration of CO_2 was determined when the stomates were closed (in the dark) as well as when they were open (in the light).

Additional studies were conducted with Hydrangea leaves in the dark. In these experiments a section from a Hydrangea leaf, one and one-half inches in diameter, was removed with a cork borer. This section was sealed with latex to the leaf cup, figure 2, with the leaf section extending to the outer edge of the ground glass flange on the cup. A small staining dish (A) filled with 0.1 *N* $Ba(OH)_2$ was sealed to the lower leaf surface. Latex was used to seal the cut edge of the leaf section and was extended over the flange of the cup and down the side of the dish so that an air tight system was obtained. The hood was darkened three hours before the experiment was started. During the experiment the gas moved from tube (D) through the cup (C) and out the second tube (E). Any carbon dioxide absorbed in the staining dish must have penetrated the leaf.

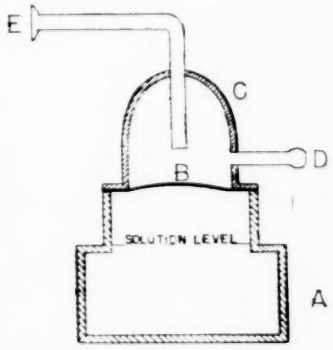

FIG. 2. Cross section of the apparatus used to measure CO_2 penetration in the dark. It consists of a leaf cup sealed to the leaf section and a dish containing the absorbing solution sealed below the leaf section.

The sampling of the leaves that were attached to the plant during the experiment was conducted in several ways. In the first method of sampling, the section of leaf under the cup was cut out with a sharp cork borer of the same diameter as the cup and the surface which had been exposed to the gas was washed with a fine stream of 0.1 *N* HCl. This was followed by several washings with distilled water. The purpose of this acid wash and rinse was to remove adsorbed carbon dioxide. The leaf section was placed between small pads of filter paper in an aluminum counting disk and dried at 80° C. A 100-gram weight was used to press the section while it was drying. A section of the treated leaf on the opposite side of the mid-vein was also pressed and dried. This section was used to measure translocation of CO_2 across the leaf. A control section was removed from the leaf on the opposite side of the stem. After the sections were dried, their activities were determined by a Geiger counter with a thin end-window.

In the second method of sampling, one treated leaf in each series of experiments was removed from the plant along with the opposite leaf and used for a gross radioautogram. The section under the cup was washed and rinsed as before but the leaf was left intact. The treated and control leaves were dried between sheets of filter paper at 80° C and placed in contact with Eastman No-Screen X-Ray film. After four weeks the film was developed and fixed and used to show the presence and distribution of C^{14}.

The detailed radioautographic technique (**3**) was also used to show the permeability of non-stomate leaf epidermis to CO_2. In the experiment with Hydrangea leaf sections as shown in figure 2, the sections under the cup were washed, dried, and counted in the usual manner. The CO_2 that penetrated the leaf was absorbed by the $Ba(OH)_2$ and precipitated as $BaCO_3$. This precipitate was transferred to a special filter disk by washing; and after drying, the activity of the carbonate was determined by a Geiger counter.

Results and discussion

In these experiments a radioactive tracer technique using C^{14} was employed to indicate absorption and movement of CO_2. Due to the low efficiency of Geiger tube counting and to the lack of standardization it is impossible to make an absolute comparison of epidermal permeability to CO_2 from the results. It is also assumed that the difference between diffusion of $C^{14}O_2$ and naturally occurring CO_2 is only about 2% as calculated from Graham's Law of Diffusion which relates the rate of gaseous diffusion to the reciprocal of the square root of the molecular weight. In an exchange reaction involving C^{12} and C^{14}, the difference in rate of diffusion would be about 10%. For a short exposure to the gas the error introduced is small. Permeability of the leaf epidermis to CO_2 has been determined by measuring the radioactivity of the carbon-14 atoms which were fixed within the leaves. Results are presented in counts per second (c/s).

Table I shows the activity of the Hydrangea leaf sections, and therefore the permeability of the epidermis to CO_2, when the leaves were exposed to 1, 5, and 10% CO_2 for four hours. The error in counting was $\pm$ 0.02 c/s and the correction for self-absorption was n/0.69. The ratio of the activities of the upper epidermis to the lower epidermis in all cases is close to unity. The absorption thicknesses of the Hydrangea and Coleus leaf sections which have been dried are 2.7 and 2.2 mg./cm.^{2}, respectively. If the C^{14} were preferentially adsorbed on the surface which had been exposed to CO_2 the ratios listed in column 4 of table I would not be one. The self-absorption of C^{14} β rays by samples of 2.7 and 2.2 mg./cm. decreases the measured activities to 0.68 and 0.74 of the maximum specific activity. Therefore, the activity of the untreated side of the leaf sections would be less than the activity of the treated side if the CO_2 were adsorbed. This would mean that the activity ratios would be greater than unity for experiments in which the upper epidermis was treated and less than unity when the lower epidermis was treated. The mean value of the ratio for Hydrangea is 1.03

$\pm$ 0.04. For Coleus the mean value is 1.05 $\pm$ 0.07. The mean values of the activity ratios as well as most of the individual observations are greater than one. This fact can be attributed to the greater photosynthetic fixation of carbon in the palisade tissue of the leaf as compared to the spongy tissue (9).

Figure 3 is a flat radioautogram of a Hydrangea leaf which was exposed to 10% CO_2 for four hours. The leaf cup was sealed to the upper epidermis. At the end of the experiment the exposed epidermis was washed with 0.1 *N* HCl and water before drying. After the leaf was dried, it was placed between two No-Screen X-Ray films for four weeks. The intensity of blackening of the two X-Ray films was the same. If the CO_2 had been preferentially adsorbed on the upper epidermis the autograph of the lower epi-

TABLE I

THE PERMEABILITY OF HYDRANGEA LEAF EPIDERMIS TO CO_2 AS MEASURED BY THE ACTIVITY OF THE LEAVES EXPOSED TO RADIOACTIVE CARBON DIOXIDE.

Per cent. CO_2	Epidermis exposed	Light or Dark	Activity of exposed epidermis c/s	Activity of upper epidermis / Activity of lower epidermis	Activity of untreated half of leaf c/s	Activity of control leaf section c/s
10	Upper	Light	7.36	1.11	0.14	0.06
10	Lower	Light	21.60	1.05	0.20	n.a.*
10	Upper	Dark	0.15	1.05	n.a.	n.a.
10	Lower	Dark	0.15	1.03	n.a.	n.a.
5	Upper	Light	2.27	1.02	n.a.	n.a.
5	Lower	Light	37.93	1.01	n.a.	n.a.
5	Upper	Dark	0.06	1.03	n.a.	n.a.
5	Lower	Dark	0.49	0.97	n.a.	n.a.
1	Upper	Light	1.53	1.00	0.11	n.a.
1	Lower	Light	48.01	0.99	0.28	0.12
1	Upper	Dark	0.15	1.02	0.10	0.08
1	Lower	Dark	0.87	1.02	0.09	0.05

* No activity.

dermis would have been less intense than the autograph of the upper epidermis.

Additional evidence that CO_2 penetrates non-stomated epidermis is shown in figure 4. Histological preparations of Coleus and Hydrangea leaves exposed to CO_2 were prepared according to the method of DUGGER and MORELAND (3). Figure 4 is a photomicrograph of a cross section of a Coleus leaf. The upper epidermis of the leaf was exposed to 1% CO_2 for four hours. The leaf section was in contact with the photographic plate for four weeks prior to developing and staining. Figure 4 shows a larger number of grains in the palisade cells of the leaf than in the spongy cells. This agrees with the activity ratio value listed in table III.

From the results of table I a comparison can be made of the relative permeabilities of the lower and upper epidermis of the Hydrangea leaf to

carbon dioxide. When the upper epidermis of the leaf is exposed to 10% CO_2 the amount of carbon fixed within the leaf is about 30% of the amount fixed when the lower epidermis of the leaf is exposed. At 1 and 5% CO_2 in the external gas the values are 4 and 6%. These small values at 1 and 5% CO_2 are due in part to the decrease in the penetration of the gas through the upper epidermis and in part to the increased penetration of CO_2 through the epidermis with stomates. The lower values for the amount of carbon fixed when absorption occurred through the lower epidermis of Hydrangea leaves at 5 and 10% CO_2 are probably caused by the influence of the gas on the stomates and the photosynthetic mechanism. HEATH (**5**) found that the CO_2 concentration of air caused a decrease in the stomate aperture. He used a porometer for determining stomate size. Carbon dioxide-free air on the other hand caused the stomates to open wider. This was in agreement with the earlier work of MASKELL (**7**), who reported that photosynthesis

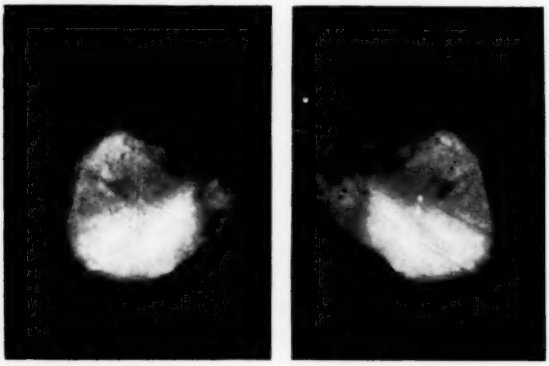

FIG. 3. Radioautograms of the upper (left) and lower (right) surfaces of a Hydrangea leaf. The cup was sealed to the upper epidermis.

was directly related to stomate movement. LIVINGSTON and FRANCK (**6**) also found that photosynthesis in Hydrangea leaves was decreased by high concentrations of CO_2. They found that the effect of a high concentration of CO_2 is strongly influenced by internal factors which are not controllable.

Table I also gives the results on lateral movement of the carbon product across the mid-vein and to the opposite leaf. At 10% CO_2 the counting data for Hydrangea leaves show that in the four-hour exposure to labeled CO_2 considerable movement across the mid-vein occurred. At 1% CO_2 the activities of the unexposed half of the leaf show considerable variations. The plants used in the experiments with 1% CO_2 had been used previously in experiments with 5 to 10% CO_2 which would account for the presence of activity in the untreated half of the leaves as well as in the control leaf sections. At the highest concentration of CO_2, the presence of activity in the control leaves indicates movement across the stem in the exposure time of four hours. RABIDEAU and BURR (**10**) found no transport of labeled

photosynthate to the opposite primary leaf of Phaseolus, and in experiments with Hydrangea leaves exposed for short periods to 1 and 5% carbon dioxide no transport was observed.

Dark fixation of CO_2 by Hydrangea leaves is a small fraction of the light fixation of CO_2. In all the experiments involving exposure of leaves to 5 and 10% CO_2 in darkness, the leaves were in darkness for one hour before exposure to CO_2; and in experiments with 1% CO_2, the leaves were in darkness for 15 minutes before exposure to CO_2. The results given in table I show that fixation in the dark is influenced by the interval of darkness before exposure to CO_2. BENSON and CALVIN (1) found this effect in Scene-

FIG. 4. Radioautogram of Coleus leaf section. The upper epidermis was exposed to 1% CO_2 for four hours. The photographic plate was exposed for four weeks.

desmus. SMITH (13) working with Helianthus, and THURLOW and BONNER (14) working with Bryophyllum, observed considerable dark fixation of tracer carbon.

From the results of table I, it is impossible to ascertain whether light influences the permeability of the epidermis. Only a small amount of carbon is fixed within the leaves in the dark as compared with the amount fixed within the leaves in the light. A series of experiments, therefore, was performed to determine whether light influences the permeability of the epidermis or only the fixation of carbon within the leaf. The results are given in table II where each value is the average of four experiments. It is evident from the results that light does not influence the permeability of

TABLE II

THE PERMEABILITY OF HYDRANGEA LEAF TO CO_2 IN THE DARK AS MEASURED BY THE ACTIVITY OF THE $BaC^{14}O_3$ PRECIPITATE FORMED BELOW THE LEAF.

Per cent. CO_2	Activity of leaf section c/s	Activity of precipitated carbonate c/s
10	0.37	21.97
5	0.08	10.32

the epidermis. The activity of the leaf section used to separate the gas from the absorbing solution is only a small fraction of the activity of the $BaCO_3$ formed, and the activity of the precipitate is proportional to the CO_2 concentration on the opposite side of the leaf. In the light, CO_2 was removed from the intercellular spaces of the leaf tissue by photosynthesis. As a result, the concentration gradient of CO_2 across the leaf epidermis was maintained. In the dark the concentration gradient of CO_2 extended through the leaf, and the permeability of the epidermis in the dark is indicated by the high activity of the precipitated carbonate.

The results obtained with Coleus are given in table III where each value is the average of duplicate experiments. The correction for self-absorption is n 0.74. The epidermis of Coleus without stomates is more permeable to

TABLE III

THE PERMEABILITY OF COLEUS LEAF EPIDERMIS TO CO_2 AS MEASURED BY THE ACTIVITY OF THE LEAVES EXPOSED TO RADIOACTIVE CARBON DIOXIDE.

Per cent. CO_2	Epidermis exposed	Light or Dark	Activity of exposed epidermis c/s	Activity of upper epidermis / Activity of lower epidermis	Activity of untreated half of leaf c/s	Activity of control leaf section c/s
10	Upper	Light	26.28	1.01	0.46	0.27
10	Lower	Light	51.44	1.08	0.20	n.a.*
10	Upper	Dark	0.14	0.02	0.04	0.04
10	Lower	Dark	0.13	1.01	n.a.	n.a.
5	Upper	Light	14.91	1.01	n.a.	0.06
5	Lower	Light	51.12	1.13	0.11	n.a.
5	Upper	Dark	0.07	1.03	n.a.	n.a.
5	Lower	Dark	0.29	1.06	n.a.	n.a.
1	Upper	Light	21.43	1.17	0.17	0.07
1	Lower	Light	20.75	1.07	0.12	n.a.
1	Upper	Dark	n.a.	....	n.a.	n.a.
1	Lower	Dark	0.13	0.99	0.05	0.05

* No activity.

CO_2 than the corresponding Hydrangea epidermis. At 10% CO_2 the Coleus epidermis is 3.5 times more permeable than the Hydrangea epidermis. At 1 and 5% CO_2 the values are 14 and 6.4 respectively. The Coleus leaves exposed on the upper surface did not show a continuous decline in the average activity with a decrease in CO_2 concentration as was observed in Hydrangea, and larger amounts of carbon were fixed by Coleus leaves at the lower concentration. The activity of the Coleus leaf sections exposed on the lower surface did not increase with decreasing CO_2 concentration as was observed with Hydrangea. At 1% CO_2 the activity of the Coleus leaf sections decreased to a value much smaller than the maximum activities obtained with leaves exposed to 5 and 10% CO_2. At 1% CO_2, the average activities of the Coleus leaves exposed on the upper and lower surfaces were almost identical. Gross autoradiograms of such leaves were also identical.

The difference in the cuticles of the Hydrangea and Coleus leaves could account for part of the observed differences in permeability to carbon dioxide. Examinations were made of the epidermal layers of leaves of the two species which showed that the upper epidermis of the Hydrangea leaf is covered with a thicker layer of cutin than the upper epidermis of Coleus. This difference, however, was small. The zone of cutinized cellulose was greater in the epidermis of Coleus. The influence of cutin on CO_2 diffusion through non-stomate leaf epidermis was investigated by FREELAND (**4**) on a variety of plant leaves. His results showed that in some plants, particularly those with a thick cuticle, little or no apparent photosynthesis could be detected when the epidermis without stomates was exposed to carbon dioxide. For other plants, including Coleus, he found that the amount of CO_2 exchanged through epidermal cells alone was approximately equal to the amount which diffused through the stomates.

The observed differences in the structure of the epidermal layers of Coleus and Hydrangea does not appear to be sufficient to account for the differences in premeability. Even though photosynthesis was not inhibited in Hydrangea by a high concentration of CO_2 (**6**) there was a reduction in the average activity at 5 and 10% CO_2. In Coleus the retarding influence of the excess CO_2 was not demonstrated.

The occurrence of dark fixation of CO_2 in Coleus leaves and the inconsistencies in translocation of the fixed carbon product to the untreated half and to check leaves were similar to the results with Hydrangea.

Summary

The permeability of the cutinized epidermal cells of Hydrangea and Coleus leaves to gaseous CO_2 was investigated by the use of radioactive carbon dioxide.

The results of the study indicate that the non-stomate cutinized epidermis of leaves of both Hydrangea and Coleus are permeable to gaseous CO_2. The degree of permeability of the epidermal layer of Hydrangea to CO_2 is proportional to the partial pressure of the gas above the epidermis. This correlation was not observed for the epidermis of Coleus. There was more penetration, however, through Coleus epidermis at 10% CO_2 than at 1 and 5%. Carbon dioxide at the higher concentrations reduced fixation of carbon in Hydrangea leaves when the stomate epidermis was exposed to the gas. This effect is probably linked with the toxic action of a high concentration of CO_2 on the photosynthetic mechanism. At 1% CO_2, the amount of CO_2 gas penetrating the non-stomate epidermis of Coleus is equal to the amount entering the leaf when the stomate side is exposed.

The results indicate that light does not influence the permeability of the leaf epidermis to CO_2.

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THE MEASUREMENT OF OXIDATION-REDUCTION POTENTIALS OF COTTONSEED SLURRIES

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(WITH THREE FIGURES)

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Introduction

The method for measuring oxidation-reduction potentials of cottonseed slurries described in this paper was developed as an additional tool for investigating deteriorative changes which occur in seeds during storage. Techniques previously employed in this laboratory (**9, 10, 17**), involving the measurements of the production of heat and of the formation of free fatty acids in the seed oil, measure only the end products of metabolic activity. In addition to data obtained by these measurements, it is desirable to learn more about the biological systems in cottonseed as affected by conditions of maturity, harvest, handling and storage, information which would be gained by studies of specific enzyme systems. As a preliminary to these studies, it was considered helpful to obtain an overall picture of the oxidizing and reducing systems in the seeds. Such an indication is given in the measurement of potentials of seed slurries under aerobic and anaerobic conditions. Patterns are obtained which show the comparative vigor of the oxidizing and reducing properties of the different lots of seeds from which these slurries are prepared.

Oxidation-reduction potentials (**6, 15**) have been used in the biological sciences to study bacterial (**14**) and yeast (**3**) metabolism, to characterize mediators of cellular oxidation (**2**), and to study plant (**1, 4, 21**) and animal (**11, 16**) tissues and fluids. Methods of obtaining potentials have involved the use of oxidation-reduction indicator dyes (**8**), direct potentiometric measurements (**19**), and combinations of both techniques (**5**). The applicability of these methods for the determination of oxidation-reduction potentials in cottonseed slurries was tested. Indicator dyes alone could not be used since the deep yellow color of the slurries masked color changes in the dyes. Moreover, in combination with the direct potentiometric methods, their contributions as mediators were not great enough to warrant their use, considering their possible activity as toxic or catalytic agents. Therefore, the method adopted involves electrometric measurement by means of bare platinum electrodes immersed in slurries, homogenized under anaerobic conditions. Details of the method are given because uniformity in the construction and care of the electrodes and in preparation of the slurry was found to be essential for obtaining reproducible results.

Methods

The slurries are prepared by homogenizing cottonseed kernels with distilled water in a Waring Blendor. Agitation by this means was found to be

satisfactory for obtaining the homogenate, notwithstanding its reported inactivation of some enzyme systems (**20**). Although from 10 to 20 grams of meats per 100 grams of cold water can be used, 15 grams of meats yield a slurry which is relatively easy to transfer and which will not foam when it is aerated.

The whole meats are added to chilled, distilled water which has been aerated for 10 minutes with nitrogen purified by passing through three chambers containing FIESER's solution (**12**). The mixture of seeds and water is aerated with purified nitrogen for an additional 10-minute period by means of a tube inserted through a rubber stopper in the cover of the blendor. Through a second rubber stopper in the cover of the blendor, a U-tube is inserted which serves both as a gas outlet during blending and later as a siphon for transferring the slurry to the measuring cell.

Following the preliminary aeration, the blendor is placed under a bell jar which is twice evacuated to 20 mm. mercury and filled with nitrogen of 99.7% purity. Without interrupting the nitrogen aeration of the slurry, the meats and water are blended for three minutes, alternating on and off at 30-second intervals. During the entire preparation, the temperature is not allowed to rise above 80° F. room temperature. This precaution is necessary because the rates of attainment of constant levels of oxidation and reduction were found to be affected by temperature changes during preparation.

Buffer solutions were not used in preparing the slurries because the natural buffering capacities of the seeds were adequate to maintain constant pH throughout the interval during which the potential measurements were made. The pH values of the various cottonseed slurries, with the exceptions of those made from seedlings, were 6.6 ± 0.2.

The slurries are kept under nitrogen until they reach room temperature at which time they are transferred by siphoning through the U-tube previously mentioned to a glass cell which is fitted with electrodes, a glass stirrer, a salt bridge, and a tube connected to a flowmeter. Measurements have been made using one electrode immersed in 50 ml. of slurry in a cell of 75 ml. capacity and eight electrodes in a cell of 500 ml. capacity containing 225 ml. of slurry. In either case the measuring cell and the calomel cell are set in a water bath maintained at 80° F.

Purified nitrogen is bubbled through the slurry at the rate of 50 ml. per minute until the readings vary less than 10 mv. in a 15-minute period. Air is then bubbled through the cell until a constant level is reached. This completes the reduction-oxidation measurements and gives a pattern of potential-time relationship which is characteristic of the material under investigation. Results are considered inconclusive unless the electrodes of duplicate determinations check within 10 mv. The potentials are referred to the normal hydrogen electrode.

No factors were found to be more important in obtaining reproducible results than the construction, cleaning, and storage of the platinum electrodes. Each electrode is made from platinum wire, 0.33 cm. in diameter,

which is silver-soldered to a brass connector and then sealed through lime glass tubing, leaving 1 cm. of the wire exposed. At the other end of the glass tubing the brass connector is secured with sealing wax to form an air-tight seal. Before each experiment the electrodes are cleaned for two minutes in hot, concentrated chromic acid, rinsed twice in hot distilled water and stored overnight in distilled water. Before being used in seed slurries, new electrodes are checked in a dilute solution of quinhydrone in phosphate buffer, pH 7.0 (0.012 grams of quinhydrone in 50 ml. M/10 buffer). Electrodes which fail to come to equilibrium within one minute when immersed in this solution are unsatisfactory for measuring the potentials of the slurries. Electrodes are also checked periodically in slurries prepared from a sample of prime cottonseed stored under conditions which minimize changes during storage. Failure to obtain checks between electrodes or failure to duplicate well established patterns previously obtained for this seed would indicate that the electrodes have become poisoned.

A Cambridge-Electronray pH meter or a Beckman pH meter is used to measure the difference in potential between a bare platinum electrode immersed in a seed slurry and a saturated calomel electrode. Electrical contact between the two half-cells is maintained by means of a bridge containing a saturated solution of potassium chloride. The platinum electrodes are joined by shielded leads to a single-pole, eight-throw switch box which is connected to the meter.

Satisfactory results are obtained with the circuit as described. However, before the construction of electrodes, their storage, and the preparation of the slurry had been so improved, the use of high resistors (50 meg.) reduced the tendency of the electrodes to become polarized by limiting the current drawn from the electrodes (7). Even though their use is no longer essential, the insertion of high resistors acts as a safeguard against polarization.

Results

In figure 1 are shown the differences in oxidation-reduction patterns obtained with two samples of prime cottonseed. One sample was from seed which was freshly harvested and the other was from a lot which had been stored at 36° F for two years. The slurry made from freshly harvested seed shows the marked resistance to oxidation which is characteristic of slurries from seed of this type.

Cottonseeds were germinated and the sprouted seeds removed from hulls and lint by hand. The curves obtained on slurries made from these seedlings are shown in figure 2. Through the third day of germination, the curves duplicated the curve obtained with the prime resting seed, although the pH dropped 0.2 unit with each day of germination. But after the fourth day of germination, the shape changed. Experiments showed that the electrodes were still sensitive at the conclusion of this test. However, after the experiment with five-day seedlings, the electrodes were found to be "poi-

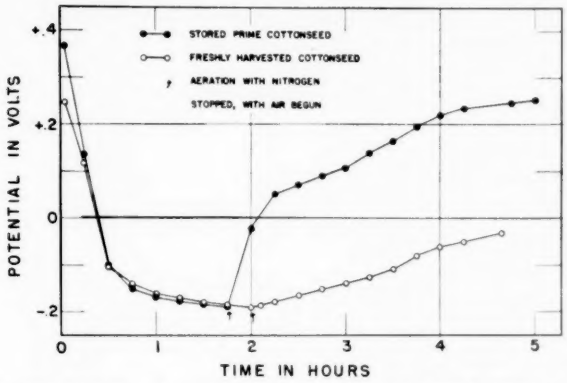

FIG. 1. Oxidation-reduction potential-time curves of freshly harvested and stored prime cottonseeds.

soned." Since neither the nature of the "poisoning" nor the point during the experiment at which it occurred has been determined, the validity of the data obtained with the five-day seedlings is questionable.

Figure 3 shows differences in the oxidation-reduction potential patterns resulting from heat and chemical treatment of the seed. The slurries were made from cottonseed of high free fatty acids content, some samples of which had been treated at 100° F with a mixture of propylene glycol dipropionate and 1,3-dimethyl-4,6-bischloromethyl benzene in the ratio of 1.5 grams of chemicals per kilogram of seed. The samples were stored for two

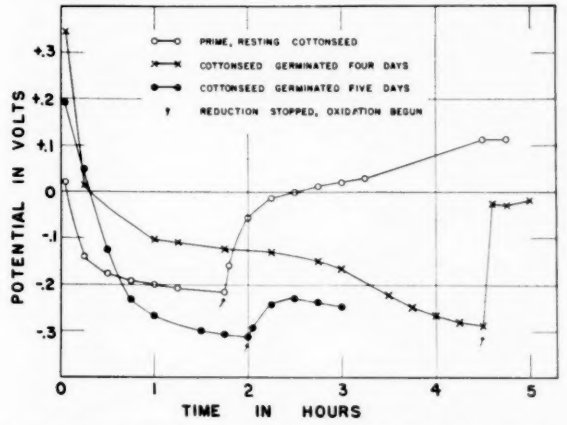

FIG. 2. Oxidation-reduction potential-time curves of prime, resting cottonseed and seedlings.

days at 100° F before the oxidation-reduction potential patterns were obtained.

That the potentials obtained are biological phenomena can be shown by the lack of reducing and oxidizing activity in an autoclaved slurry. It has also been established that the proliferation of microörganisms is not responsible for the activity because the patterns are not altered when sufficient sodium ethyl mercuri-thiosalicylate is added to prevent their growth.

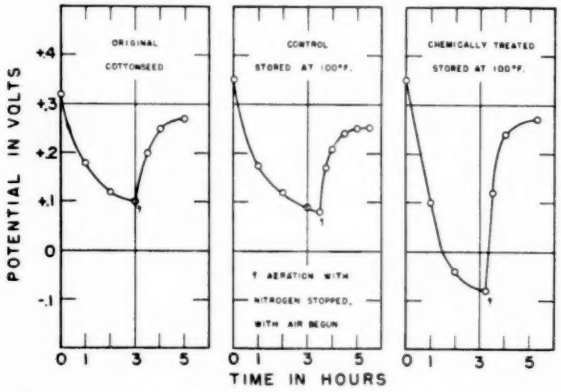

FIG. 3. Oxidation-reduction potential-time curves of deteriorated cottonseed; control, heated, chemically treated and heated.

Discussion

While measuring the potentials of the slurries, it was found that despite careful construction and handling, electrodes become insensitive or produce erratic results. With slurries of prime, resting cottonseed, this change occurs after approximately 20 experiments. The usual cleaning method is ineffective, and recovery is obtained only by flaming which is usually equivalent to the construction of a new electrode. This "poisoning" of the electrodes is not due to a mechanical fault such as the failure of the glass to platinum seal because the same "poisoning" is obtained after only one experiment with new electrodes in a slurry made from seeds germinated five days. It would seem to be due to some biological substance which increases in concentration during germination. FREEDMEN and CORWIN (**13**) experienced difficulties with platinum electrodes in measuring the potentials of thiol compounds and attributed it to formation of a Pt-thiol compound on the surface of the electrode.

It is clear that this method is sensitive in detecting differences in oxidizing and reducing properties of various lots of seed as manifested in the shapes of the oxidation-reduction potential-time curves. For example, two lots of seed with low free fatty acids and moisture contents and approxi-

mately equal viability, differing only in age, gave widely different patterns (fig. 1). At this time the patterns have principally empirical value; no explanations are offered for the differences obtained. The difficulties cited by MICHAELIS and FLEXNOR (**18**) in the application of the concept of definite redox potentials to physiological materials in which many processes are irreversible apply to the interpretation of the data presented here. Additional work on the nature of the electrode reaction in slurries of cottonseed of known history and on the components that contribute to the potential will allow further interpretation of the data.

Summary

A method for measuring oxidation-reduction potentials of cottonseed slurries is described. The importance of exercising care in the construction and cleaning of the platinum electrodes and the necessity of preparing the slurry under purified nitrogen are emphasized.

Platinum electrodes, sensitive for approximately 20 experiments with resting seed, are "poisoned" after only one experiment with five-day cotton seedlings.

The potential-time patterns obtained with cottonseed slurries vary with chemical treatment and length of time of germination, as well as with the age of the seed.

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THE RELATION OF ACIDS AND PEROXIDES IN SPRAY OILS TO THE RESPIRATION OF SPRAYED BEAN LEAVES AND THE DEVELOPMENT OF INJURY

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(WITH FOUR FIGURES)

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The tremendous expansion in the use of weed-killing chemicals in recent years and especially the development of oils which have various degrees of selective action upon different kinds of plants, have led to considerable interest in the nature of the toxic ingredients and the biochemical disturbances which they cause. The fundamental discovery of Gray and deOng (**10**) that the phytotoxicity of spray oils is measured more or less quantitatively by the percentage reduction in volume upon treatment with hot, concentrated sulphuric acid (the so-called UR test) was followed by Tucker's (**24**) suggestion that the portion of the oil removed in the UR test is less important than the acidic substances to which it gives rise during exposure to light and air either before or after application to the plant. He attributed the toxic effects to the oil-soluble asphaltogenic acids of high molecular weight, probably the same as those described by Staeger (**22**). Injury to apricot leaves kept in darkness after application of oil was found only if the content of asphaltogenic acids was at least 0.5% by weight. Hence the toxic effect was due to a very minor constituent of the oil rather than the whole sulphonatable fraction which was as high as 50% in some of Tucker's oils.

The distinction between rapid or acute injury caused by light oils and slow or chronic injury resulting from the presence of heavy oils as first made by deOng *et al.* (**7**) was elaborated by Crafts and Reiber (**5**) who classified injury into four categories: (a) acute injury from volatile unsaturates, *e.g.*, benzene, Edaleanu extract and certain constituents of gasoline or kerosene; (b) acute injury from volatile acidic compounds formed in low-boiling unsaturates, *e.g.*, in diesel and heavy fuel oils; (c) chronic injury from high-boiling unsaturates, *e.g.*, in diesel and heavy fuel oils; and (d) chronic injury from non-volatile acidic compounds formed in high-boiling distillates upon exposure to light and air. Type (d) was that studied by Tucker.

The so-called "Superior" spray oils consist of approximately two parts saturated paraffin compounds to one part saturated naphthenic compounds (**4, 20**). High insecticidal efficiency and minimum plant injury have been claimed for them. However, these oils upon exposure to light develop acid-

ity and become markedly phytotoxic though the UR is not changed appreciably (**11**). Plant injury may be correlated more or less quantitatively with the amount of acid formed. A point of much interest is the suggestion by CRAFTS and REIBER (**5**) that acute plant injury may result from peroxides formed in refined oils exposed to light and air during storage. There appear to be no data available on the comparative effects upon plant tissue of acids and peroxides in spray oils when present singly or together.

It has been known for a long time that both peroxides and acids occur in hydrocarbons such as those of refined spray oils after they have been subjected to oxidizing conditions, but the relation, if any between them was uncertain. By analysis of refined lubricating oils kept at 250° F (121° C) DENISON (**6**) showed that after an induction period the peroxide concentration rose rapidly, passed through a maximum and then fell to a low and persistent value. Simultaneously the acidity rose, at first at an increasing rate and then less rapidly. He concluded that peroxides are formed first and are an essential step in the course of oxidation since the rate of uptake of oxygen was proportional to the peroxide concentration at all times. The buildup in acidity followed but was somewhat below the oxygen absorption. Agreement with this theory has been expressed in recent reviews by GEORGE and ROBERTSON (**9**) and by ZUIDEMA (**27**) who pointed out that rupture of the carbon chain at the point where a hydroperoxide is first formed accounts for both the non-volatile acids and the short chain acids such as formic, acetic, etc., found by FENSKE and co-workers (**8**).

Since quantitative data on the relative importance of peroxides and acids in plant injury from spray oils are very meager in the literature, it seemed worthwhile to apply a number of oils containing these materials at known concentrations to plants raised and held in controlled conditions. Some preliminary experiments showed that tissue respiration was affected strongly, so the toxicity study was accompanied by the determination of changes in respiratory activity caused by application of the same oils.

Previous reports on the effect of spray oils upon the respiration of leaves have been scanty and somewhat confusing. KELLY (**16**) dipped leaves of Grimes apples into oil emulsions and found examples of both increased and decreased respiration after a few hours, as measured by CO_2 output. GREEN and JOHNSON (**13**) found that detached bean leaves increased their CO_2 output after being sprayed with dark oils having over 16% sulphonatable constituents, whereas similar tests with refined oils having less than 16% sulphonatable constituents resulted in a decrease. The idea that oil may decrease respiration by plugging the stomata and perhaps some of the interior passages was discounted by GREEN (**12**) because he found that the effects just described were not altered by variation in the amount of oil applied. However, YOUNG (**26**) emphasized the difference between chemical effects which lead to increased respiration and physical plugging of stomata and intercellular spaces with resulting decrease in respiration. It may be noted that all of the investigators used leaves with their natural

content of respirable materials which varied of course with the previous exposure of the plants.

Materials and methods

Two highly refined spray oils (H and 23) consisting almost entirely of saturated aliphatic and naphthenic compounds and containing no detectable peroxide or acid were taken as starting materials. These oils and those prepared from them by laboratory oxidation were supplied together with specifications by the California Research Corporation. They correspond in composition fairly closely to the "Superior" oils mentioned earlier. Oxidation by the technique of DENISON (**6**) gave the following derived oils: (a) oil 81 with high peroxide and low acid content from short oxidation followed by treatment with alkali, (b) oil 36 with medium high acidity and fairly low peroxide content from short oxidation followed by treatment with reducing agent, and (c) oil 80 with high acid and low peroxide content from long oxidation followed by treatment with reducing agent. A number of other derived oils were obtained by exposing the refined oils in a thin film to sunlight and air for one to seven days during the summer. One aromatic oil (A) was used in a few comparative tests. The characteristics of all these materials are given in tables I and II. Acidity was determined by the official method (**1**) using 0.02 *N* NaOH for the titration. Peroxides were determined by WHEELER's method (**25**).

TABLE I

PHYSICAL AND CHEMICAL PROPERTIES OF UNOXIDIZED OILS USED IN PHYTOTOXICITY AND RESPIRATION TESTS.

Code number	UR	Sulphur	Saybolt viscosity		Paraffins	Naphthenics	Aromatics	Acids	Peroxides
			100° F	210° F					
	%	%			%	%	%	*m.eq./kg.*	*m.eq./kg.*
H	99.8	0.03	57.0	34.2	61	39	0	0.0	0
23	99.7	0.01	56.3	33.9	60	40	0	0.0	0
A	81.6	0.41	59.6	34.3	64	22	14	0.5	0

Dwarf horticultural bean plants, *Phaseolus vulgaris*, reared in the greenhouse in soil or in Hoagland's nutrient medium were used for both injury and respiration tests. They were used when about four weeks old, when several full sized leaves had developed. The oils were applied undiluted by means of an atomizer until both upper and lower surfaces were covered without appreciable runoff. Deposits determined by weighing immediately before and after spraying averaged approximately 20 mg./200 mg. leaf weight. This unit is used to facilitate comparison with deposits in the respiration tests. The deposit was purposely made greater than that used in practical spraying in order to exaggerate the toxic effects. Applications were made in the mornings of sunny days. The temperature cycle in the

greenhouse was fairly uniform, the maximum near midday usually lying between 90 and 100° F. Observations of injury were made at intervals up to several weeks in some cases, with special attention to the effects upon young mature leaves.

Since respiration of plant tissue is affected considerably by variation in the amount of soluble carbohydrate present, all plants were kept in the dark for 24 hours before use in these tests. Middle leaflets of young mature bean leaves were removed from several plants and cut into squares, 2 mm. × 2 mm., which were then mixed thoroughly. Samples weighing 200 mg. (fresh weight) were taken and sprayed with the chosen oil until covered with a film. Excess oil was blotted up. The final ratio was about 60 mg. oil per

TABLE II

PHYSICAL AND CHEMICAL PROPERTIES OF OXIDIZED OILS USED IN PHYTOTOXICITY AND RESPIRATION TESTS.

Code number	Deviation	Acids	Peroxides
		m.eq./kg.	*m.eq./kg.*
36	Oil H oxidized at 130° C, then treated with reducing agent	110	30
80	Oil 23 oxidized at 130° C, then treated with reducing agent	225	10
81	Oil 23 oxidized at 130° C, then washed with alkali	10	310
	Oil A in thin film exposed to sunlight for 0 days	0.5	0.0
	6 days	3.1	2.0
	Oil H in thin film exposed to sunlight for 0 days	0.0	0.0
	6 days	1.1	3.4
	Oil 81 in thin film exposed to sunlight for 0 days	10.0	180.0*
	1 day	24.0	190.0
	4 days	64.0	205.0

*This sample remained in the laboratory for several months during which time the peroxide content declined from 310.

200 mg. of leaf squares. Each sample was put into a 15-ml. Warburg vessel together with 2 ml. of 1% glucose in 0.1 M phosphate buffer solution at pH 4.5. Vacuum infiltration according to the method of BONNER and WILDMAN (**2**) was then carried out to facilitate penetration of both oil and nutrient. Gas exchange was measured in the dark at 26° C. The Warburg vessel contained 5% potassium hydroxide solution in the center well and was moved through 120 oscillations per minute. Measurements of respiratory activity were started within one hour after spraying. Certain manometric measurements of the activity of respiratory enzymes were carried out by the Warburg technique, the details being stated later in connection with specific tests. Reducing sugars and sucrose extracted by boiling 80% alcohol were determined by the ferricyanide-ceric sulphate method of HASSID (**14, 15**).

Results

Visible effects from the oils

Application of oils to intact bean plants under greenhouse conditions resulted in varying degrees of injury to the plants depending on the acidity, and, to a lesser extent, the peroxide concentrations in the oils. In figure 1, the range of injurious effects is illustrated. Oil H, a neutral, non-peroxidic oil caused no visible changes in the appearance of the plant for several days. After about three weeks, leaves which had been sprayed with oil H were slightly yellow but no necrotic spotting was evident. Highly acidic oils caused severe injury within two to three hours and in 24 hours the whole plant was dead. Since oil H was innocuous, it was used as a diluent for the toxic oils in order to study their effects in more detail. Thus 10% oil 36 (by volume in oil H) caused no injury to bean leaves in four hours,

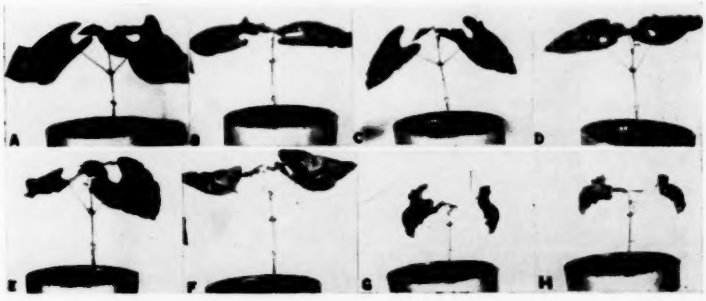

Fig. 1. Effects of petroleum oils on bean leaves kept under greenhouse conditions. A. Controls. B. Oil H, after 24 hours. C. 10% oil 36 after four hours (acid 11 m.eq./kg. and peroxides 3 m.eq./kg.). D. 10% oil 36, after 24 hours. E. 100% oil 81 after four hours (acids, 10 m.eq./kg. and peroxides 310 m.eq./kg.). F. 100% oil 81 after 24 hours. G. 100% oil 80 after four hours (acids 230 m.eq./kg. and peroxides 10 m.eq./kg.). H. 100% oil 80 after 24 hours.

but slight wilting and many localized necrotic spots were apparent in 24 hours (plants C and D of fig. 1). After three weeks the leaves appeared normal except for some small dead spots. Dilution to 2 and 1% eliminated all evidence of harmful effect within several days. Oil 81, the high-peroxide, low-acid oil, when applied undiluted to the plants caused only slight injury in four hours; but within 24 hours the leaves had developed a dry, leathery texture with some necrotic tissue at the margins. After several days, the leaves had recovered except for dead spots; but if the plants were kept in the dark, the withered condition persisted. When diluted to 10%, this oil produced no visible injury until several days had elapsed. Plants G and H illustrate the rapid and extensive injury resulting from the application of highly acidic oils, in this case oil 80. Similar responses to undiluted oil 36 were also obtained. The leaves were severely injured soon after the oil was applied. Complete death of the plant occurred in less than 24 hours.

In order to distinguish, if possible, between plant injury caused by acids in the oils and injury due to peroxides, a further experiment was conducted with the three toxic oils, each diluted with oil H to give a uniform acidity of 5 milliequivalents per kilogram. The necessary concentrations were 2.5% oil 80, 5% oil 36, and 50% oil 81. Figure 2 shows typical results from the various treatments. No injury could be observed within the first few hours; but 24 hours later, diluted oils 80 and 36 each caused slight darken-

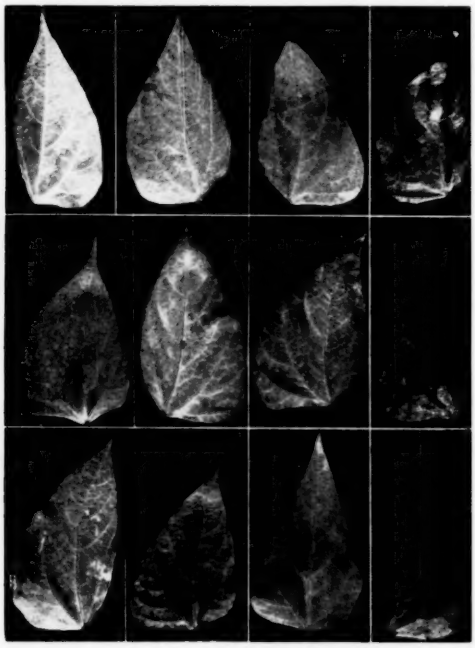

FIG. 2. Effect of peroxides in petroleum oils on bean leaves. Acidity of all oils constant at 5 m.eq./kg. Top: 24 hour exposure under greenhouse conditions. Middle: 96 hour exposure under greenhouse conditions. Bottom: 96 hour exposure in continuous darkness. Left to right in each row: control, 2.5% oil 80, peroxides 0.25 m.eq./kg.; 5% oil 36, peroxides 1.5 m.eq./kg.; 50% oil 81, peroxides 155 m.eq./kg.

ing in a few areas whereas diluted oil 81 caused considerably greater darkening with some leathery areas. Thus 5 milliequivalents acid per kilogram appears to be near the lower limit for production of obvious signs of injury within 24 hours except when much peroxide is also present. After 96 hours under normal greenhouse conditions some recovery had occurred. Plants treated with the oils and kept in the dark to prevent photosynthesis were in a worse condition. It is obvious that the presence of a large concentration of peroxide was associated with greater injury after one or more days.

The toxic oils mentioned so far had been oxidized in the laboratory at an elevated temperature and hence would not necessarily have effects similar to those of oils exposed to light and the atmosphere at ordinary temperatures. The data of table II show that several oils low in both acid and peroxide increased several fold in each during the course of a week's exposure in a thin film in a flat graniteware pan. When these were then sprayed upon bean leaves, the resulting injuries corresponded closely to those already described for the artificially oxidized oils when diluted to equal acidities. Thus oil H (acidity after exposure, 1.1 m.eq./kg.) caused some yellowing in a week to 10 days and oil A (acidity after exposure, 3.1 m.eq./kg.) caused barely detectable darkened spots in 24 hours.

Effects of oils upon oxygen uptake of plants and leaves

Since the published reports indicate an uncertain relation between the visible acute injury caused by spray oils and changes in the respiratory activity of plants, some tests were made with complete bean plants sprayed with oils A and H before and after exposure in thin films. Only carbon dioxide output was measured. While oil H had no detectable effect, the oxidized oils showed both repression and stimulation of carbon dioxide production in a manner which seemed dependent upon previous exposure of the plant to darkness and time after spraying, but the behavior of control plants was too variable to permit quantitative measurement of changes without much more elaborate experimental arrangements.

It appeared much more worthwhile to attempt the measurement of the respiration of leaf segments under standardized conditions after treatment with the various oils. Placing the bean plants in darkness for 24 hours brought them to a fairly uniform condition since analyses of young mature leaves showed the reducing sugar content to average 0.14% and total sugars to average 0.22% with variations only in the second decimal. Oxygen uptake was affected inappreciably by vacuum infiltration of buffer solution, typical results being 52 and 53 mm.^{3} O_2/200 mg. tissue/hour for leaf segments suspended in the buffer and infiltrated, respectively. Similar runs on the same lot of leaf segments with 1% glucose present showed slightly higher activity, *i.e.*, 58 and 61 mm.^{3} O_2/200 mg. tissue/hour. The increase signified that depleted leaves are able to utilize added glucose but the process of infiltration made no large difference in response. Very young leaves respond twice as rapidly. Probably most variation in behavior in the various tests is attributable to difficulty in selecting leaves of the same age. Oxygen uptake is affected appreciably by the pH of the suspending medium. Thus, in a series of tests, the average rate over three hours (in mm.^{3} O_2/200 mg. tissue/ hour) was 69 (pH 4.5), 69 (pH 5.5), 59 (pH 6.5), 58 (pH 7.5), and 54 (pH 8.5). All other tests were maintained at pH 4.5 by use of a 0.1 M phosphate buffer.

Leaf segments not treated with oil maintained a steady respiration for several hours. Different samples varied appreciably despite efforts to treat

all plants alike and to select leaves at the same stage of development. Thus among 53 lots of leaf segments used as untreated controls the range of oxygen uptake was 43 to 73, averaging 62 mm.^{3} O_2/200 mg. tissue/hour. A similar variation occurred among lots of treated leaves, *e.g.*, among five lots treated with 10% oil 81, the range was 39 to 47, averaging 42 mm.^{3} O_2/200 mg. tissue/hour. However, for each oil a portion of the same lot of leaf segments was used as control and variation among samples drawn from within the same lot was much smaller. Thus seven typical pairs used with 10% oil 36 gave the following uptake of oxygen (average for three hours, in mm.^{3} O_2/200 mg. tissue/hour): controls, 63, 69; 60, 60; 59, 63; 61, 64; 58, 60; 69, 72; 63, 66; and treated, 28, 30; 23, 26; 24, 28; 26, 28; 18, 19; 39, 41; 18, 22. The marked consistency of both control and treated samples from within each leaf lot accounts for the comparatively small standard deviations of the means, or standard errors, shown in table III. It should be pointed out, however, that the early (15 to 30 minutes) measurements of the effects of 10% oil 36 were much more variable.

TABLE III

EFFECT OF VARIOUS OXIDIZED OILS AND MIXTURES OF THEM WITH OIL H UPON OXYGEN UPTAKE OF LEAF SEGMENTS.

Oil	Acidity	Peroxides	Number of determinations	Reduction in oxygen uptake compared with control leaves	Calculated reduction from linear regression equation
	m.eq./kg.	*m.eq./kg.*		%	
H	0.0	0.0	38	4 ± 0.9*	10.8
1% oil 36	1.1	0.3	10	7 ± 2.2	16.5
3% oil 36	3.3	0.9	9	15 ± 3.5	26.8
5% oil 36	5.5	1.5	13	47 ± 4.5	37.2
10% oil 36	11.0	3.0	112	55**	....
1% oil 80	2.3	0.1	8	25 ± 3.6	29.7
2.5% oil 80	5.6	0.3	4	35 ± 2.1	36.9
5% oil 80	11.3	0.5	8	71 ± 3.9	64.0
10% oil 80	22.5	1.0	8	88 ± 3.6	> 100.0†
10% oil 81	1.0	31.0	20	14 ± 2.2	21.9
25% oil 81	2.5	77.5	4	50**	....
50% oil 81	5.0	155.0	12	67**	....
100% oil 81	10.0	310.0	9	85 ± 1.9††	97.0

*Standard error of mean.

**This value at one hour continued to fall; not used in calculating regression equation.

†This value is meaningless since 100% is maximum reduction possible.

††Oxygen uptake continued to fall but slowly enough to permit a mean figure to be used.

The oils which quickly caused visible injury, *e.g.*, 100% oil 36 or oil 80, stopped respiration too soon to permit measurements. Ten per cent. oil 36 and 25 and 50% oil 81 caused a continuing decrease in rate of oxygen uptake for several hours. A few tests with 20% oil 81 showed the same continuing decrease but leaf segments treated with the other oils lowered their

respiration rapidly and within one half to one hour assumed a steady state which persisted for several hours. The experimental data illustrating the characteristic types of behavior are shown in figure 3 for 10% oil 36, 10% oil 80, and oil H.

Similar plots for the other oils have been used to determine the average reductions in rate of oxygen uptake. The data are given in table III, column 5. The standard errors of the mean calculated from all measurements for each treatment except early ones before the steady state was reached are included to indicate the reliability of the data. For 10% oil 36 and

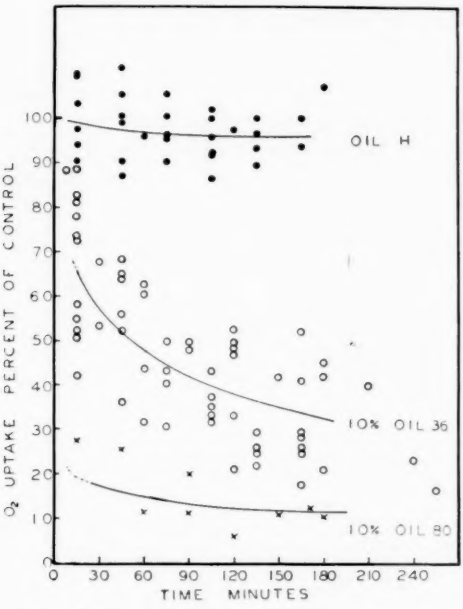

FIG. 3. Effects of oils on the oxygen uptake of bean leaf pieces. Oil H, neutral, non-peroxidic oil; 10% oil 36, acidity 11 m.eq./kg., peroxides 3 m.eq./kg.; 10% oil 80, acidity 23 m.eq./kg., peroxides 1 m.eq./kg.

25 and 50% oil 81, the values at one hour are given for purposes of comparison, though no steady state was reached; and hence a standard error would not have the same meaning as with the other oils. It appears that an acid content of about 1 m.eq./kg. is needed to depress oxygen uptake beyond the slight effect of oil H. Hence repression of respiration is a more delicate index of physiological action than visible injury and may occur to a considerable degree without subsequent ill effects to the leaves.

Since the oils included in table III vary widely in their content of acids and peroxides and in the ratio between these constituents, it is possible to

calculate their relative importance in reducing the oxygen consumption rate. The simplest procedure is to regard reduction in rate of oxygen absorption (Y) as a simultaneous linear function of acid content (X_1) and of peroxide content (X_2), the two latter being expressed in milliequivalents/kg. of oil. The method of least squares may be used to calculate the best fitting straight line having the general equation: $Y = b_{y1.2}X_1 + b_{y2.1}X_2 + \text{constant}$ [Equation 1, nomenclature of SNEDECOR (**21**), page 340], in which

$$b_{y1.2} = \frac{(\Sigma X_2^2)(\Sigma X_1 Y) - (\Sigma X_1 X_2)(\Sigma X_2 Y)}{(\Sigma X_1^2)(\Sigma X_2^2) - (\Sigma X_1 X_2)^2}$$

and

$$b_{y2.1} = \frac{(\Sigma X_1^2)(\Sigma X_2 Y) - (\Sigma X_1 X_2)(\Sigma X_1 Y)}{(\Sigma X_1^2)(\Sigma X_2^2) - (\Sigma X_1 X_2)^2}$$

Substituting the respective sums of squares and cross products calculated from table III, it will be found that $b_{y1.2} = 4.66$, $b_{y2.1} = 0.13$ and constant = 10.8. The equation then becomes, $Y = 4.66\,X_1 + 0.13\,X_2 + 10.8$ (Equation 2). The oils which did not cause a fairly steady rate of oxygen uptake were not used in the calculation.

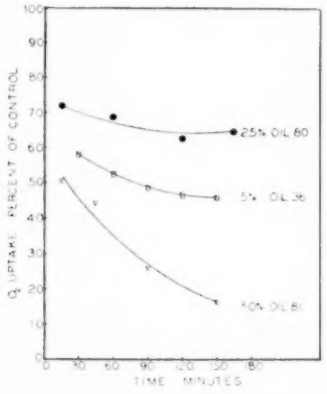

FIG. 4. Effect of oils of equal acidity on the oxygen uptake of bean leaf pieces. Acidities constant at 5 m.eq./kg. Peroxides variable as follows: 2.5% oil 80, 0.25 m.eq./kg.; 5% oil 36, 1.5 m.eq./kg.; and 50% oil 81, 155 m.eq./kg.

The relative effects of acids and peroxides upon oxygen uptake within a few hours after application of the oils are measured by the coefficients of X_1 and X_2 and hence acids are more effective than peroxides in the ratio 4.66 : 0.13 or approximately 36 to 1. The last column of table III gives reductions in rate of oxygen uptake calculated from Equation 2. The average disagreement with experimental valves is 8.2 units, and the order of increasing effect is the same for the actual and the calculated data. The equation assumes the effects of acids and peroxides to be proportional to the

first power of their concentrations over the whole range represented, possibly other equations might represent these relations more accurately; but the present method of calculation shows unmistakably that the acids found in oxidized spray oils have much more effect on respiration of bean leaves than the peroxides have. Since early visible injury as described and illustrated earlier runs parallel to depression of oxygen uptake as shown in table III, it follows that the acids are several fold as important as peroxides in causing acute injury.

It was mentioned in describing injury from the three oils of equal acidity, *i.e.*, 5% oil 36, 2.5% oil 80, and 50% oil 81, that increasing effects with time resulted from application of the last oil which contained a large concentration of peroxides. Similarly, in the case of leaf segments treated with 50% oil 81 the oxygen uptake did not decrease to a relatively steady state but continued to fall for several hours. The data for the three oils are shown in figure 4. It may be noted that 10% oil 36 is somewhat unique in that it caused a continuing decline in rate of oxygen uptake which only appeared to reach a steady state near the end of the three hour observation period although the peroxide content was fairly low.

TABLE IV

RESPIRATION OF LEAF SEGMENTS TAKEN FROM BEAN PLANTS AT INTERVALS AFTER SPRAYING WITH OIL.

Oil	Interval after spraying	Oxygen uptake in mm.^{3} O_2/200 mg. tissue/per hour		
		Control	Sprayed	% of control
	hrs.			
5% oil 80	3	114	81.5	71
	26	82	107	130
	50	83	97	117
25% oil 81	3	104	86	83
	26	90	93	103
	50	104	128	123
100% oil 81	3	94	17*	18
	26	61	27	44
	50	61	17	28

*No steady state was reached within the initial period of measurement but the value at one hour is given to show magnitude of the effect.

Three oils were used in a study of oxygen uptake over longer periods. Greenhouse bean plants were sprayed as in the determination of visible injury except that one half of each plant was covered during application of the oil. Leaves were taken from each half at once and after 24 and 48 hours, cut into squares, 2 mm. × 2 mm., and infiltrated with the customary buffered glucose solution. Average results of three tests over three hour periods for each interval after spraying are given in table IV. Steady values persisted over the periods of measurement except in case of 100% oil 81 immediately after spraying.

Leaves treated with 5% oil 80 and 25% oil 81 recovered from the initial reduction in oxygen uptake but 100% oil 81 caused a permanent reduction doubtless because of the dead areas which developed in the sprayed leaves. The super normal oxygen uptake one and two days after application of the two less harmful oils probably represents an acceleration of activities after the initial shock. It cannot be due to unknown differences in the plants or vagaries of the weather since the controls were the unsprayed halves of the same plants and also their oxygen uptake was comparable to that of leaves from untreated plants measured at the same time. It may be noted that nearly all values of the controls in table IV are higher than the 43 to 73 $mm.^3$ O_2 found among the controls used in the measurements of leaf squares sprayed with oils which is to be expected since the plants which were sprayed before the leaves were cut into squares received about one third as much oil per 200 mg. leaf and they were not depleted of nutrients by prolonged storage in darkness. Also, infiltration of oil through the leaf structure doubtless is much more complete when the integument has been partly destroyed in the process of cutting up the leaves.

Effects of oils upon certain respiratory systems

Some limited efforts to ascertain how acidic oils depress the oxygen uptake of bean leaf segments were largely unsuccessful but they may be mentioned briefly. Addition of several organic acids known to play a part in respiration of yeast and animal cells caused an increase in oxygen uptake in the absence of oils. When added at the rate of 10 mg. acid to 200 mg. leaf tissue in 2 ml. glucose solution at pH 4.5, the following increases in rate of oxygen uptake occurred: succinic acid, 24%; pyruvic acid, 22%; citric acid, 15%; malic acid, 12% and fumaric acid, 5%.

In contrast, malonic acid at 1 mg./ml., and at pH 4.5, reduced oxygen uptake by 37% and at 5 mg./ml. reduced it by 77%, while output of carbon dioxide was reduced by 8 and 74%, respectively. Succinic acid at 1 mg./ml. completely reversed the inhibitory effect of malonic acid at 1 mg./ml. but caused only a slight restoration of respiration when malonic acid was present at 5 mg./ml. Increasing succinic acid to this level caused little further change, possibly because the total salt concentration was then nearly 0.2 M and salt effects became important (**17**). Fumaric acid also inhibited the effect of malonic acid but less effectively. This behavior is similar to that which occurs in cells of animals and certain microörganisms and has been explained in terms of inhibition of succinic dehydrogenase by malonic acid and reversal of this inhibition by excess succinic acid. Hence it was of interest to determine if the acids of oxidized spray oils acted similarly. The results obtained by adding various oils and succinic acid to bean leaf segments are given in table V.

Succinic acid accelerates oxygen uptake when oil H is present just as it does with unsprayed leaf segments and it has almost as strong an effect with 10% oil 81 but the oxygen reduction caused by the various other more

acidic and more injurious oils is not affected, although a large excess was used. By contrast succinic acid overcame the effect of malonic acid at a 1 : 1 ratio. Hence it appears that the oxidized oils do not act specifically in the portion of the respiratory process in which succinic, fumaric and other related acids and their corresponding enzymes are concerned, *i.e.*, in a cycle resembling the Krebs' cycle of other organisms.

The respiration of bean leaf segments prepared in the usual manner is inhibited by hydrogen cyanide to the following extent, the first and second figures denoting reduction of oxygen uptake and carbon dioxide output compared with a normal control: 0.0001 M HCN, 13 and 3%; 0.001 M HCN, 21 and 15%; 0.01 M HCN, 64 and 61%. Since this behavior is not specific for any one terminal oxidase system, but is a general one indicating some heavy metal ion enzyme, further tests were made by adding ascorbic

TABLE V

EFFECT OF SUCCINIC ACID UPON UPTAKE OF OXYGEN BY BEAN LEAF SEGMENTS SPRAYED WITH VARIOUS SPRAY OILS. ONE PER CENT. GLUCOSE SOLUTION IN PHOSPHATE BUFFER AT pH 4.5 INFILTRATED INTO LEAF SEGMENTS.

Oil	Acid added in oil	Succinic acid added	Ratio oil acid / succinic acid	Oxygen uptake in mm.$^3O_2$/ 200 mg./hr.		
				Control	Oil	Oil and succinic acid
	m.e.	*m.e.*				
Oil H	0	85×10^{-3}	....	53	52	70
5% oil 36	0.33×10^{-3}	85×10^{-3}	1/260	57	26	27
10% oil 36	0.66×10^{-3}	170×10^{-3}	1/260	60	26	22
1% oil 80	0.14×10^{-3}	85×10^{-3}	1/600	57	43	45
10% oil 81	0.06×10^{-3}	85×10^{-3}	1/1400	53	47	62
50% oil 81	0.3×10^{-3}	85×10^{-3}	1/280	57	20	28
100% oil 81	0.6×10^{-3}	85×10^{-3}	1/140	53	12	11

acid to leaf segments and to leaf tissue breis both with and without added hydrogen cyanide. In no case was there any effect indicating the presence of ascorbic oxidase in the leaf tissue.

Evidence for the existence of polyphenol oxidase in bean leaf tissue was also inconclusive. The characteristic transient rise in oxygen utilization by catechol-treated tissue, followed by a pronounced depression of oxygen uptake observed by BOSWELL and WHITING (**3**) and by BONNER and WILDMAN (**2**) could not be demonstrated either with infiltrated bean leaf fragments or with leaf breis. BONNER and WILDMAN (**2**) regard *p*-nitrophenol as a rather selective inhibitor of polyphenoloxidase and, as the result of demonstrations of both *p*-nitrophenol inhibition and the characteristic respiratory responses to catechol, conclude that polyphenol oxidase is present in spinach leaves. In the present experiments, bean leaf respiration was inhibited 80% by 0.001 M *p*-nitrophenol. However repeated failure to demonstrate the catechol effect makes it seem unlikely that polyphenol oxidase makes an

important contribution to the terminal oxidase activity of young mature bean leaves. STENLID (**23**) has suggested that *p*-nitrophenol is an inhibitor of rather general effect upon oxygen consumption and that it probably reacts with several enzymes rather than selectively on polyphenol oxidase.

Cytochrome oxidase appears to be the most active terminal oxidase in the bean leaf tissue studied as shown by measurements on breis prepared from leaf fragments in a blendor. The endogenous respiration of a brei (equivalent to 0.5 gm. fresh leaf in 2 ml. at pH 7) was 8 mm.^{3} per hour. Addition of 0.9 to 9.0 mg. cytochrome c and 6 mg. hydroquinone resulted in an increase in oxygen absorption to an average level of 59 mm.^{3} per hour. Hydrogen cyanide at 0.002 M completely eliminated the increase in oxygen consumption. Heat-inactivated brei plus the addenda showed an oxygen absorption of 8 mm.^{3} per hour. This behavior in the presence of these substrates, supported by failure to demonstrate either polyphenoloxidase or ascorbic oxidase, strongly suggests that cytochrome oxidase is the most active oxidase in these leaves.

Addition of 0.2 ml. of 100% oil 36 to 2 ml. of the brei plus cytochrome c and hydroquinone had no effect upon oxygen uptake. Hence it may be concluded that the oxidized oils do not interfere with bean leaf respiration through inhibition of the terminal cytochrome oxidase. It may be noted that 0.2 ml. of 100% oil 36 applied to the equivalent of 0.5 gm. fresh weight of leaves is far in excess of that which completely inhibits the respiration of intact leaf tissue. Intimate contact of the oil with the enzyme-bearing leaf fragments was assured by the rapid oscillation of the Warburg vessels.

EFFECTS OF OXIDIZED OILS UPON REDUCING SUGAR–SUCROSE BALANCE IN BEAN LEAVES

Respiratory enzyme poisons have been found to inhibit synthesis of sucrose from glucose supplied to barley shoots (**18**). Hence it was of interest to determine if oxidized spray oils had a similar effect with bean leaves. Plants were depleted of carbohydrates by storage in darkness for 22 hours, after which time samples in the form of disks three fourths of an inch in diameter were removed from representative leaves. These were well mixed and used in groups of 10 per test. Oils were applied as a spray in approximately the same amounts as in the respiration studies, *i.e.*, 60 mg./200 mg. leaf weight, and 5% glucose or sucrose solution was infiltrated under vacuum. Treated samples were then kept in darkness for 24 hours. External sugar was removed by careful rinsing before analysis. A number of tests with oil 36 added to sucrose solutions and shaken for periods up to 24 hours proved that no hydrolysis is affected by the acids or other constituents of the oil.

Table VI shows that the ability of bean leaf segments to form sucrose decreases as more acidic oils are added. Repression of sucrose formation is characteristic of the oils which inhibit respiration and the situation resembles that in respiration-inhibited barley shoots. Oil H and 10% oil 36 promote sucrose formation possibly by changing permeability conditions.

TABLE VI

EFFECTS OF OILS UPON BALANCE BETWEEN REDUCING SUGARS AND SUCROSE IN BEAN LEAVES. ALL PLANTS LEFT IN DARKNESS 22 HOURS BEFORE SPRAYING WITH OIL AND VACUUM INFILTRATION OF SUGAR SOLUTION. LEAF SEGMENTS KEPT IN DARKNESS FOR 24 HOURS AFTER TREATMENT.

Treatment	Reducing sugars	Sucrose	Reducing sugars / Sucrose
Depleted leaves	0.14	0.08	1.8
No oil, 5% glucose infiltrated	3.18	0.28	11.3
Oil H, 5% glucose infiltrated	2.07	0.68	3.0
10% oil 36, 5% glucose infiltrated	2.28	0.56	4.1
25% oil 36, 5% glucose infiltrated	3.07	0.12	25.6
50% oil 36, 5% glucose infiltrated	3.11	0.09	34.6
100% oil 36, 5% glucose infiltrated	3.46	0.05	69.2
No oil, 5% sucrose infiltrated	0.72	3.38	0.21
Oil H, 5% sucrose infiltrated	0.72	3.12	0.23
10% oil 36, 5% sucrose infiltrated	1.08	2.10	0.52
25% oil 36, 5% sucrose infiltrated	1.30	2.09	0.62
50% oil 36, 5% sucrose infiltrated	1.28	2.24	0.57
100% oil 36, 5% sucrose infiltrated	2.21	2.00	1.11

On the other hand, infiltration of sucrose into leaves treated with acidic oils resulted in accumulation of reducing sugars. The acidic oils apparently have little or no effect on the invertase catalyzed hydrolysis of sucrose. However, the same oils markedly inhibit the synthesis of sucrose from reducing sugars, whether infiltrated or arising from hydrolysis of sucrose, and the net result is a shifting of the dynamic balance between mono and disaccharides in the direction of an accumulation of reducing sugars at the expense of the infiltrated sucrose.

Discussion

The rapid effects of oxidized spray oils derived from highly purified aliphatic and naphthenic stocks upon the appearance and the respiration of plants are closely correlated with content of acids, whereas peroxides give rise to more slowly appearing changes of the same nature. The differences might be explained in terms of slower penetration of the peroxides; but the experimental fact that oils containing much peroxide rapidly form further amounts of acid upon exposure in thin films indicates that after application to plants the same process probably ensues, and the newly formed acids are the chief toxic agents. This theory is consistent with the continuing decline in rate of oxygen uptake of leaves treated with oil 81 as shown in figure 4. The injurious constituents are not simple aliphatic acids, because the application of synthetic acid oils prepared by dissolving aliphatic acids in oil H (final concentration of 10 m.eq./kg.) to bean leaves never resulted in foliage injury in greenhouse tests. Some of the synthetic acid oils caused inhibition of respiration of bean leaf pieces in the usual respiration trials. Acids causing inhibition of respiration but no foliage injury were, n-valeric, iso-valeric, n-caproic, n-heptylic, and pelargonic. Acids causing neither

foliage injury or inhibition of respiration were acetic, lauric, myristic, palmitic, oleic and adipic. It may be noted that TUCKER (**24**) found linoleic acid at 2% (71.5 m.eq./kg.) only slightly harmful to apricot leaves. Since oxidation of petroleum oils forms a preponderance of complex acids (**27**), simple ones could not be present at 10 m.eq./kg. in any of the highly injurious oils used. Analysis showed that the acids of the oxidized oils contained three to ten oxygen atoms in addition to those in the carboxyl group. Hence alcohol, aldehyde, ketone, or ether radicals were present. Some of the groups would aid in polymerization to the asphaltogenic acids discussed by STAEGER (**22**) and by TUCKER (**24**) and to the sludge or varnish found upon exposure in a thin film.

Mechanical exclusion of oxygen from respiratory sites by oils has been postulated by YOUNG (**26**) as a possible cause of reduction of respiration by oils. It is significant, however, that large excesses of neutral, non-peroxidic oils cause only a slight decrease in oxygen absorption by the tissues. Oxidized oils in similar amount caused large decreases. Oxidized oil constituents such as acids have some water solubility and would tend to collect at interfaces where changes in permeability and changes in cell-enzyme substrate relations might be induced.

The present work has contributed only negative information on the question of how the inhibition of respiration is accomplished. Bean leaves react to some of the acids concerned in Krebs' cycle and addition of these acids does not overcome the respiratory inhibition caused by the oxidized oils. There is considerable evidence that cytochrome oxidase is an important terminal oxidase in bean leaves, although probably not the only one. The situation may be similar to that found by MARSH and GODDARD (**19**) where young carrot leaf respiration is inhibited by HCN but that of mature leaves is not. Whatever the relative importance of the various terminal systems in the bean, they are not affected by oxidized oils. Positive information on the important problem of how and at what step these acids exert their effect upon respiration is still to be obtained.

A possible explanation for the lessened synthesis of sucrose in bean leaves treated with oxidized oils is that the decreased respiration gives less energy for the series of phosphorylations involved in the formation of sucrose. The more direct process of hydrolysis controlled by invertase still continues as shown by the increasing amount of reducing sugars after infiltration of sucrose. The general picture of semistarvation in leaves suffering from treatment with oxidized oils suggests that many other changes in amount and distribution of cellular constituents may be concerned.

Summary

Highly refined petroleum oils, containing no aromatics, and oxidized oils prepared from the neutral stocks were used in phytotoxicity studies on bean leaves.

Visual injury to bean leaves was best correlated with acidity of the oxidized oils. The lower limit of acidity of the oils associated with acute in-

jury of bean leaves was near 5 m.eq./kg. High peroxide concentrations resulted in evidence of injury after longer periods of exposure.

A more sensitive measure of injury was the reduction in oxygen absorption of bean leaf fragments when treated with the oils. Neutral, non-peroxidic oils had only a small effect on absorption of oxygen by the leaf fragments. The effects of oxidized oils on oxygen uptake measured immediately after application of the oil can be approximately expressed by the equation $Y = 4.66\,X_1 + 0.13\,X_2 + 10.8$, where Y is the percentage reduction in oxygen absorption, X_1 is the acidity of the applied oil expressed in milliequivalents per kilogram, and X_2 is the peroxide concentration of the oil also expressed as milliequivalents per kilogram of oil.

The respiration of intact bean plants, sprayed and kept in the greenhouse for an additional 24 or 48 hours, recovered from the initial depression and was stimulated above that of the controls provided injury was not too extensive. Malonic acid inhibits the respiration of unsprayed bean leaves and the malonate inhibition is reversed by succinate. Respiration of unsprayed and also neutral oil sprayed bean leaves is enhanced by the addition of succinic, fumaric, malic, and pyruvic acids and also by glucose. Inhibition of respiration induced by oxidized oils is not reversed by any of these substrates, indicating that oxidized oils do not specifically inhibit the succinic dehydrogenase system as, for example, malonic acid does. Evidence that cytochrome oxidase may be a component of one of the terminal oxidase systems is based on the following facts, (a) leaf respiration is inhibited approximately 60% by 0.01 M HCN, (b) ascorbic acid oxidase and polyphenol oxidase activities were negligible or absent, (c) oxygen utilization by bean leaf breis was greatly accelerated by the addition of cytochrome c and hydroquinone as substrates. A highly oxidized oil had no effect on the *in vitro* activity of the cytochrome oxidase system of bean leaf brei.

Oxidized oils which reduced the respiration of bean leaf tissue to 30% or less of the control also prevented the synthesis of sucrose from glucose infiltrated into bean leaves. This result is to be expected on the basis of reduced energy available from respiratory activity. The oxidized oils do not inhibit the hydrolysis of infiltrated sucrose.

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INHIBITION OF GROWTH BY BENZENE HEXACHLORIDE ISOMERS AND PROTECTIVE EFFECT OF GLUCOSE AS MEASURED BY CELL COUNTING TECHNIQUE

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(WITH FOUR FIGURES)

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Introduction

The purpose of this study is to evaluate the effects of benzene hexachloride isomers on root growth and the antagonism of these effects by selected chemical compounds. Interest has developed in gamma benzene hexachloride because it is highly toxic to insects (**40**). Five isomers of benzene hexachloride have been isolated (**19**) and the structural properties of most of them are well understood (**40**). The gamma and delta isomers cause mitotic arrest at metaphase (**8, 9, 13, 28, 30**). The toxicity of the gamma isomer, known commercially as Lindane, has been studied extensively (**8, 14, 18, 23, 28, 34, 38**), but little work has been done on the delta isomer (**8, 38**). Benzene hexachlorides possess certain properties characteristic of typical narcotic compounds, two of which are the lack of polar groups in the molecule and the limited solubility in water. Owing to the lack of polar groups, it would be predicted that benzene hexachloride (BHC) molecules would accumulate preferentially in the lipid phase boundaries of the protoplast (**2**). Following such accumulation, changes in cell structure and organization may occur which greatly influence the direction and rate of chemical reactions (**13, 33**).

One of the first reported antagonists of gamma benzene hexachloride was *meso*-inositol (**9, 12, 22, 27**). Earlier, this finding was of interest because of the supposed structural analogy between gamma benzene hexachloride and the biologically important inositols. It now appears, however, that the delta isomer, and not the gamma isomer, is isomorphous with *meso*-inositol (**17**). D'Amato (**12**), on the other hand, reported a delaying action of *meso*-inositol on the C-mitotic and C-tumor action of gamma benzene hexachloride, which occurred at 15 to 19° C, but not at 26 to 27° C. D'Amato also observed a delaying action of sucrose and other sugars and ascribed these effects to a change in permeability to the BHC. Antagonisms of this type are suggestive of those observed against x-radiation by sodium cyanide (**3**), thiourea (**31**) and vitamin P (**41**).

Levan and Östergren (**30**) have discussed the mechanism of C-mitotic action at some length. These workers describe C-mitotic action as a narcosis of those factors which govern growth by cell division, and C-tumor action as a narcosis of those factors which govern growth by cell tension. C-tumor formation has a threshold separate from C-mitosis (**37**). Mc-

ELROY (**33**) has presented a unifying interpretation of the mechanism of inhibition of cellular activity by narcotics, *in vivo*. He stressed that narcosis consists fundamentally of the inhibition of the Pasteur effect. McElroy's interpretation is quite useful in that it encompasses most theories heretofore advanced on the mechanism of narcosis. Furthermore, it aids materially in explaining why diverse inhibitors of cellular function appear to affect a variety of processes in somewhat the same way, even though the actual site of combination of the inhibitors in cells may be different.

Materials and methods

The rooting medium adopted after preliminary study was the special crepe paper called Kimpack, often used by seed technologists. Pieces of Kimpack eight inches square and 0.3 inch thick were placed in waxed cardboard boxes eight and one half inches square and one and one half inches deep. The required amount of BHC was pipetted from the diluted stock solution into a dispenser fitted with an aluminum sprinkler head and acetone containing 0.08% Tween 20 was added to give a volume of 130 ml. which was adequate for thorough impregnation. The treated papers were dried in a forced draft oven at 30° C for eight hours. Since BHC will volatilize under these conditions, it is imperative to maintain a constant drying time if comparable data are to be obtained between experiments. The initial BHC concentration is calculated from the volume of water used to moisten each piece of dried Kimpack and the weight of BHC added. Purified alpha, beta, gamma and delta isomers of BHC were used.

Plant material consisted of *Cucurbita pepo* (L.), variety Early Yellow Prolific squash, and *Carthamus tinctorius* (L.), Nebraska Hybrid, number 852. Previous work indicated squash roots to be sensitive to a large number of chlorinated hydrocarbon insecticides and related compounds (**25**).

All experiments were carried out in a dark room at a temperature of 27 ± 1° C and a relative humidity of about 50%. Seedlings with primary roots 1 to 3 mm. long were transplanted to treated and untreated papers previously moistened with 130 ml. of aerated tap water, then covered with moist paper toweling. A uniform planting and harvesting schedule was adhered to so as to avoid the variability associated with mitotic cycles (**21**). All experiments were terminated 48 hours after transplanting. Twenty-five seedlings were removed from the treated papers and root tips 10 mm. long were excised under water. The root tips were preserved in glacial acetic acid because standard killing solutions did not permit good maceration following transfer to chromic acid. The over-all length of primary roots was then recorded, allowing for the portion removed.

A composite sample of five root tips was placed in one ml. of 5% chromic acid and macerated after standing six hours, according to the method of BROWN and RICKLESS (**7**). Ten to fifteen root tips were studied in each treatment. Using a special pipette, a small aliquot of the macerated root suspension was transferred to the counting chamber of a Spencer

Bright-Line haemacytometer for counting of the cells. The volume of the chamber was 0.9 cubic millimeter. The microscope used for counting had a 35 mm. Leitz objective, 20 × compensating oculars and no condenser. The slide was illuminated with a Spencer microscope lamp fitted with a blue Corning glass filter, number 429. Counts were completed within 24 hours after transfer of root tips to the chromic acid in order to avoid difficulties with disintegration of the cells. The total number of cells and the number of non-vacuolated cells were determined in treated and untreated root tips. By means of this technique BROWN and RICKLESS (**7**) determined the effect of mineral nutrients, sugar and yeast extract on the rate of cell division in excised roots of *Cucurbita pepo* (L.) during a period of 12 hours. They determined the rate of cell division by dividing the average increment in number of cells by the average number of non-vacuolated cells in the root during the interval. A modification of this procedure has been used in this work. The number of non-vacuolated or meristematic cells in squash seedling root tips was found to remain constant during the four 12-hour intervals of a 48-hour experiment. The total number of cells, however, decreased progressively during this time. The number of vacuolated cells, obtained by taking the difference between total and non-vacuolated cells, likewise decreased. The assumption is made that during the first 48 hours, the dominant process in squash roots is cell division.

The ratio between non-vacuolated and vacuolated cells in untreated root tips during a period of 48 hours is plotted in figure 2. This ratio is termed the index of growth. It is of interest that the shape of the curve is similar to that of the classical growth curve. The cell counting technique is quantitative; and measures change in the combined processes of cell division, differentiation, and organization. MASCRE' and DEYSSON (**32**) used a mitotic index for comparing the effects of various chemicals on mitosis in the onion root. The average number of cells and the number of non-vacuolated cells between duplicate samples from the same bottle could be counted with an error of 5% as claimed originally for this method (**7**). A coefficient of variability of 15% was observed among 25 random samples for counts of cells. For counts of non-vacuolated cells the coefficient of variability was 20% among 25 random samples.

The extent of protection exhibited by various chemical compounds against concentrations of benzene hexachloride isomers inhibiting cell division was studied. The following compounds were dissolved in water and applied in concentrations ranging from 10^{-6} to 10^{-2} M to Kimpack paper treated with BHC: acetylcholine chloride, adenylic acid, beryllium sulphate, cysteine chloride, desoxyribose nucleic acid and ribose nucleic acid, dimethylamine, D-glucose, D,L-glyceraldehyde, ethanol and calcium chloride mixtures, *i*-inositol, iodoacetic acid, magnesium chloride, octanoic acid, phloridzin, sodium arsenate, sodium arsenite, sodium diethyldithiocarbamate, sodium fluoride, sodium pyrophosphate, thiamine hydrochloride and 2,4-dinitrophenol (**10, 15, 24, 27, 29, 42, 44**). All solutions were adjusted to

a pH of 6.0 with the exception of iodoacetic acid and sodium diethyldithiocarbamate which were used at a pH of 5.0 and 7.5, respectively. The effect of these compounds on the growth of squash roots was determined in preliminary experiments.

Inorganic and labile phosphate analyses were made on root tips essentially according to the method of KALCKAR (**26**). The method permits the determination of inorganic phosphorus in the presence of labile phosphate esters. Excised root tips weighing 500 mg. were cooled quickly to −5° C, then transferred in 10 ml. of ice cold trichloroacetic acid to a Potter-Elvehjem homogenizer (**43**) made of stainless steel. The final concentration of the trichloroacetic acid was 5%. During homogenization the stainless steel tube was kept in ice. The cold homogenized suspension was centrifuged for one to two minutes at 12,000 RPM in an angle-head centrifuge. The tube was placed in ice and a 0.5 ml. aliquot of the supernatant liquid was pipetted into 1.0 ml. of 0.1 *N* sodium acetate, and 3.0 ml. of sodium acetate–acetic acid buffer, pH 4, was added. This sample was analyzed for inorganic phosphate. Labile phosphate was determined on another aliquot which was incubated at 30° C for 30 minutes before neutralizing with the acetate–acetic acid buffer. The phosphate color was developed in both samples by adding 1.0 ml. of ammonium molybdate reagent at a concentration of 1% in 0.05 *N* sulphuric acid, and 1.0 ml. of ascorbic acid at a concentration of 1% in water. Absorption of light was measured with a Beck-

TABLE I

ENZYME INHIBITORS WHICH AFFECT THE GROWTH OF ROOTS OF *Cucurbita pepo*.

Name of compound	Concentration (molarity)	Mean total cells per root ($\times 10^3$)	Mean non-vacuolated cells per root ($\times 10^3$)	Index of growth	Mean root length
					mm.
Sodium arsenite	2.3×10^{-6}	87.4	44.0	1.01	64
	2.3×10^{-5}	80.4	38.9	0.94	61
	4.5×10^{-5}	87.2	44.4	1.04	57
2,4-Dinitrophenol (DNP)	4.2×10^{-6}	59.0	27.1	0.85	71
	4.2×10^{-5}	56.7	17.3	0.44	25
	4.2×10^{-4}	61.4	17.5	0.40	12
Sodium arsenate	2.5×10^{-6}	84.6	36.2	0.75	58
	1.2×10^{-5}	69.3	24.4	0.54	44
	2.5×10^{-4}	52.4	14.6	0.39	25
Iodoacetate	4.1×10^{-6}	86.1	39.8	0.86	75
	4.1×10^{-5}	87.0	43.6	1.00	66
	4.1×10^{-4}	73.5	32.9	0.81	51
	1.0×10^{-3}	64.7	12.8	0.25	26
Beryllium sulphate	7.3×10^{-6}	83.2	38.1	0.84	46
	7.3×10^{-5}	74.4	35.3	0.90	39
	7.3×10^{-4}	65.0	35.0	1.17	48
	7.3×10^{-3}	62.8	22.0	0.54	38
Untreated		82.0	38.7	0.90	70

mann spectrophotometer at 700 millimicrons, after the color had developed for 10 minutes.

The phosphate analyses were made to compare the effect of 2,4-dinitrophenol (DNP), which causes the release of inorganic P, with that of benzene hexachloride. Determination of labile phosphate, although specifically a measure of adenosine triphosphate (ATP), may be interpreted as such if a marked decrease in labile P occurs in the presence of the stimulant (**33**).

Results

It seemed desirable to characterize the capacity of squash roots to respond to selected chemical compounds, many of which are considered rather specific inhibitors of enzyme systems. Indices of growth for five compounds having inhibitory effects on root growth are shown in table I. Sodium arsenite and iodoacetate reduce sulfhydryl groups, and sodium arsenate and DNP act to uncouple oxidative phosphorylation (**4**). Sodium arsenate may also inhibit adenosine triphosphatase. Beryllium sulphate is considered to be an inhibitor of the prosthetic group of adenosine triphosphate (**15**). Sodium arsenite at a concentration of 2.3×10^{-4} M stopped root

TABLE II

EFFECT OF BENZENE HEXACHLORIDE ISOMERS ON TWO PLANT SPECIES.

Species	Isomer and dosage	Mean total cells per root ($\times 10^3$)	Mean non-vacuolated cells per root ($\times 10^3$)	Mean root length	C-tumor*
				mm.	
Cucurbita pepo	alpha BHC 1.1×10^{-3} M	62 ± 7	9.3 ± 4	32 ± 12	++
	beta BHC 1.1×10^{-3} M	78 ± 19	36.0 ± 6	82 ± 37	None
	gamma BHC 1.1×10^{-3} M	66 ± 38	1.6 ± 0.8	14 ± 1.9	+++
	delta BHC 1.1×10^{-3} M	44 ± 0.5	1.8 ± 0.04	10 ± 1.9	+
	None	67 ± 6	34.0 ± 0.3	75 ± 12	None
Carthamus tinctorius	alpha BHC 2.2×10^{-3} M	96 ± 9	45.0 ± 14	44 ± 12	None
	beta BHC 2.2×10^{-3} M	68 ± 7	33.0 ± 8	44 ± 18	None
	gamma BHC 2.2×10^{-3} M	84 ± 30	16.0 ± 5	26 ± 11	+
	delta BHC 2.2×10^{-3} M	46 ± 4	3.3 ± 3	17 ± 8	+
	None	84 ± 10	44.0 ± 6	67 ± 12	None

*+ = slight C-tumor, ++ = intermediate C-tumor, +++ = pronounced C-tumor.

growth in the first 12 hours of the experiment. Lower concentrations of arsenite were without effect. Sodium arsenate, iodoacetate and DNP lowered the growth index and over-all root lengths at concentrations of 10^{-3} M or lower. Beryllium sulphate inhibited root growth at all concentrations used, but the rather high concentration of 7.3×10^{-3} M was required to lower the growth index. The most striking effect on cell numbers is produced by DNP and sodium arsenate. Glyceraldehyde, phloridzin, sodium fluoride, acetylcholine, sodium diethyldithiocarbamate, and sodium pyrophosphate were without effect on root growth. These results indicate that the cell counting technique is useful in judging the growth inhibiting effects of water soluble chemicals.

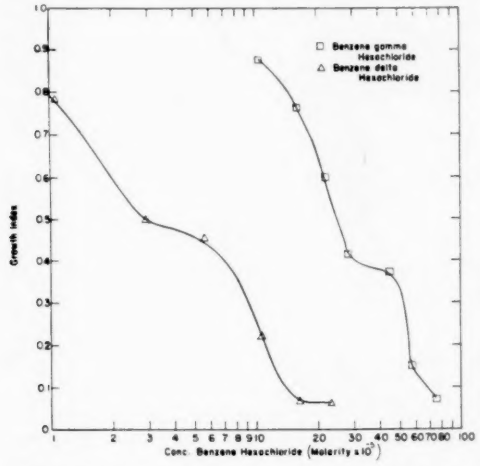

FIG. 1. Dosage response curves for the effect of gamma and delta benzene hexachloride on the growth of *Cucurbita pepo* roots.

Detailed screening experiments using four isomers of benzene hexachloride were carried out on squash, *Cucurbita pepo* (L.), and a safflower hybrid, *Carthamus tinctorius* (L.). Results are shown in table II. The C-tumor which appears in response to the gamma isomer is quite pronounced. The condition known as C-tumor arises from an unimpeded tension in all directions tending to make the root cells isodiametric (**30**).

The effect of varying concentrations of the gamma and delta isomers on the growth index is shown in figure 1 in which the logarithm of the concentration of benzene hexachloride is plotted against the growth index. Comparison of the curves shows that the concentration at which the gamma isomer produced a 50% lowering of the growth index was about fivefold higher than that for the delta isomer.

Results from an experiment in which roots of squash were maintained

in contact with various concentrations of gamma benzene hexachloride for periods of 14 to 48 hours are shown in figure 2. The sharply rising index of growth between 24 and 36 hours in untreated roots reflects the large number of non-vacuolated cells in proportion to vacuolated cells. High concentrations of BHC abolish this condition. It is apparent from the curve for the untreated roots that the growth index approaches unity at 48 hours.

Results from an experiment in which squash roots were placed in contact with gamma benzene hexachloride for 2 to 36 hours and then transferred to untreated media for recovery in a 48 hour experiment are shown in table III. These data reveal an interaction of time and concentration. It is also evi-

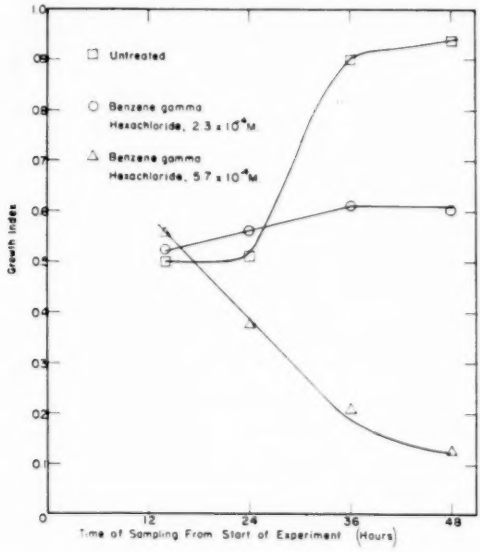

FIG. 2. Time course of the growth index for untreated and treated roots of *Cucurbita pepo* seedlings.

dent that the growth index decreases rather uniformly with increasing time of contact with the exception of the contact periods of 14 hours at the higher dosages.

Among the 22 compounds studied, D-glucose was the most effective antagonist of both the gamma and delta isomers of benzene hexachloride as shown in table IV. The lengths of untreated roots, however, exceeded those of the roots grown in glucose and BHC. Glucose gave no protection against a BHC concentration of 5.7×10^{-4} M. Dinitrophenol at 10^{-4} M with a concentration of BHC producing a C-tumor, 5.7×10^{-4} M, abolished the reaction(s) responsible for the production of the tumor, but the over-all growth was suppressed. The inhibitory effect of the combination of BHC with DNP was the same as if either compound had been used singly.

TABLE III

CELL NUMBERS, ROOT LENGTHS, AND GROWTH INDEX, AS AFFECTED BY VARIOUS CONTACT INTERVALS AND CONCENTRATIONS OF GAMMA BENZENE HEXACHLORIDE.

BHC dosage	Contact time	Recovery time	Mean total cells per root ($\times 10^3$)	Mean non-vacuolated cells per root ($\times 10^3$)	Mean root length	Growth index
	hrs.	*hrs.*			*mm.*	
2.3×10^{-4} M	2	46	90 ± 3	42 ± 4	90 ± 12	0.88
	6	42	81 ± 9	37 ± 2	79 ± 11	0.84
	14	34	62 ± 22	24 ± 15	64 ± 10	0.63
	24	24	82 ± 32	31 ± 16	47 ± 7	0.61
	36	12	61 ± 20	22 ± 14	40 ± 10	0.56
3.4×10^{-4} M	2	46	89 ± 14	36 ± 13	83 ± 11	0.68
	6	42	88 ± 10	36 ± 16	75 ± 12	0.69
	14	34	83 ± 20	40 ± 16	68 ± 9	0.93
	24	24	82 ± 10	32 ± 2	46 ± 8	0.64
	36	12	64 ± 10*	23 ± 12	39 ± 8	0.56
5.7×10^{-4} M	2	46	76 ± 20	33 ± 4	88 ± 18	0.77
	6	42	67 ± 1	23 ± 5	79 ± 10	0.52
	14	34	73 ± 19	43 ± 11	62 ± 8	1.43
	24	24	59 ± 28*	20 ± 16	40 ± 8	0.51
	36	12	46 ± 6*	8 ± 2	25 ± 6	0.21
None	....	....	68 ± 5	33 ± 1	57 ± 10	0.94

*Evidence of C-tumor.

TABLE IV

PROTECTION EXHIBITED BY GLUCOSE AGAINST INHIBITORY EFFECTS OF GAMMA AND DELTA BENZENE HEXACHLORIDE.

Concentration BHC isomer	Concentration of glucose	Mean root length	Growth index	Percentage protection*
		mm.		%
gamma BHC:				
2.8×10^{-4} M	2.1×10^{-4} M	50 ± 13	0.87	132
2.8×10^{-4} M	2.1×10^{-4} M	45 ± 9	0.55	54
2.8×10^{-4} M	4.2×10^{-4} M	52 ± 15	0.79	112
2.8×10^{-4} M	None	44 ± 13	0.33	....
None	4.0×10^{-4} M	75 ± 10	0.93	....
None	None	73 ± 13	0.74	....
delta BHC:				
2×10^{-4} M	4.3×10^{-4} M	37 ± 18	0.42	31
2×10^{-4} M	4.3×10^{-4} M	26 ± 9	0.42	31
2×10^{-4} M	4.3×10^{-4} M	54 ± 15	0.86	88
2×10^{-4} M	None	25 ± 12	0.18	....
None	4.0×10^{-4} M	73 ± 10	0.90	....
None	None	70 ± 10	0.95	....

*$\frac{\text{Growth index for BHC and Glucose Combination} - \text{Growth index for BHC}}{\text{Growth index for Untreated} - \text{Index for BHC}} \times 100.$

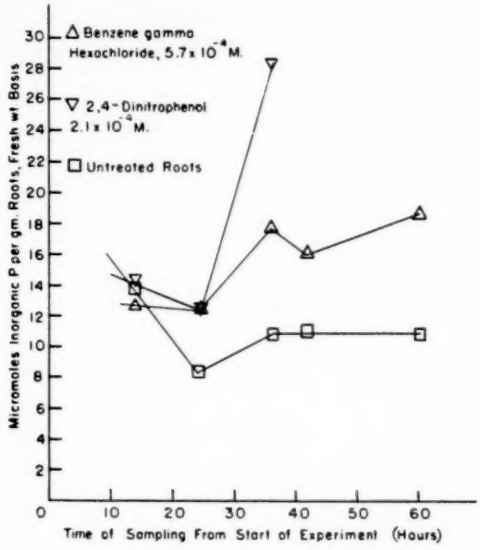
Micromoles Inorganic P per gm. Roots, Fresh wt Basis
Time of Sampling From Start of Experiment (Hours)
Benzene gamma Hexachloride, 5.7 x 10⁻⁴ M.
2,4-Dinitrophenol 2.1 x 10⁻⁴ M.
Untreated Roots

FIG. 3. Time course of the release of inorganic phosphate in untreated and treated roots of *Cucurbita pepo* seedlings.

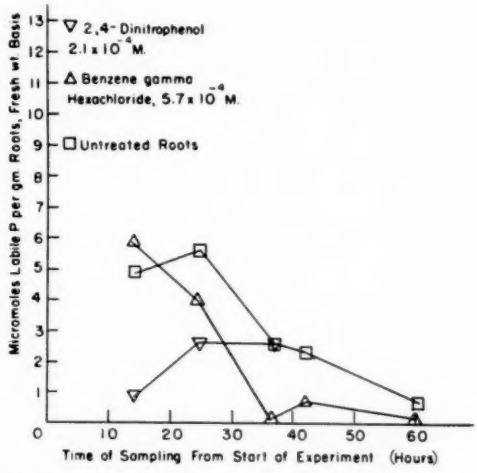
Micromoles Labile P per gm. Roots, Fresh wt Basis
Time of Sampling From Start of Experiment (Hours)
2,4-Dinitrophenol 2.1 x 10⁻⁴ M.
Benzene gamma Hexachloride, 5.7 x 10⁻⁴ M.
Untreated Roots

FIG. 4. Time course of the decrease in acid-labile phosphate in untreated and treated roots of *Cucurbita pepo* seedlings.

Analyses for inorganic and labile phosphate in trichloroacetic acid extracts of squash roots grown in contact with DNP and BHC for varying periods of time are shown in figures 3 and 4. Analyses of untreated roots for the same time periods are also shown. Presence of DNP causes an accelerated release of inorganic P between 24 and 36 hours. A parallel release of inorganic P is caused by BHC. The amount of labile phosphate in the roots treated with DNP for 14 hours was much less than the amount in the untreated roots. During further treatment the labile phosphate increased and at 36 hours was equal in amount to that in the untreated roots. On the other hand, treatment with BHC caused a sharp decrease in the amount of labile phosphate and at 36 hours there was little or no labile phosphate present. These results are in agreement with what would be predicted for the effect of stimulants in the concept advanced by McElroy (**33**).

Discussion

The cell counting technique adapted from Brown and Rickless (**7**) is useful in judging the inhibitory effects of both water-soluble and water-insoluble compounds on growth. The striking effects of DNP, arsenate, arsenite, and iodoacetate on the growth of squash roots shown in table I reveal the dependence of the processes of cell division and differentiation upon enzyme systems concerned in the mobilization of energy (**13**). Both DNP and arsenate are considered as stimulants whose action ultimately may abolish the Pasteur effect (**33**). The inhibitory effect of sodium arsenite and iodoacetate reveals the presence in squash roots of sensitive enzyme systems containing sulfhydryl groups. It is not clear, however, whether the —SH groups which arsenite specifically reduces are part of the proteins in the mitotic spindle, or whether these groups are parts of the enzyme systems concerned in mobilization of energy for the mitotic process (**13**). Dustin (**16**) believes that cell division is dependent upon a concentration of —SH groups in the cell which must be maintained within narrow limits. Nickerson and VanRij (**35**) suggest that two groups of chemicals can poison the mitotic process. One group includes the water-soluble chemicals which are specific for sulfhydryl groups and directly affect cell division. The second group is the water-insoluble narcotics which affect the formation of new, discontinuous phase boundaries at the end of cell division.

Rather specific effects of isomers of benzene hexachloride on root growth are suggested by the data in figure 1, by virtue of the narrow range of concentrations over which they act. The nature of these effects cannot be predicted on the basis of the experiments carried out here. It is suggested, however, that the presence of benzene hexachloride molecules in phase boundaries of the protoplast may initially disorganize the lipid component and cause the disorganization of the chain of respiratory processes (**2, 35**). The results in table II reveal a variation between species in the growth response to some of the BHC isomers. In squash roots the alpha isomer

lowers the number of non-vacuolated cells and causes C-tumor formation, but in safflower roots neither response was observed. The assumption that lipid soluble compounds denature proteins by changing the organization of lipid residues in the polypeptide chains making up the proteins allows for variation among species (**13, 37**). Within a given species, a difference in the spatial configuration of the molecule is associated with a difference in response. The molecular shape of a given isomer may determine the amount of the compound which can penetrate the root (**1, 33**). Reactions may occur at the surface of the cell, however, which obviate the necessity of penetration (**24, 35, 44**).

Data in table III indicate that development of the C-tumor in the highest concentration of BHC is accompanied by a large decrease in the total number of cells and the number of non-vacuolated cells. This observation is at variance with the observations of LEVAN and ÖSTERGREN (**30**) who found that in the tumor induced by colchicine the total number of cells remains unchanged. Possibly the BHC tumor is structurally unlike that induced by colchicine. The increase in the growth index at 14 hours for the highest concentration of BHC in table III is in sharp contrast to the rather low index for the treatment with 2.3×10^{-4} M BHC. This condition arises from the increased number of non-vacuolated or meristematic cells at 14 hours induced by 5.7×10^{-4} M BHC. BLUMENTHAL (**6**) has reported an increase in mitotic activity in some tissues during phases of an imposed starvation period.

The C-tumor appears 12 hours earlier in the treatment with 5.7×10^{-4} M BHC than in the 3.4×10^{-4} M treatment. This situation seems analogous to that reported by NORTHEN for colchicine (**36**), in which the concentration and time of contact were of importance in causing the dissociation of labile spindle proteins. In general, the data in table III indicate irreversible effects of BHC at all concentrations after 14 hours of contact.

The effect of glucose at 4×10^{-4} M in protecting against growth inhibiting concentrations of the gamma and delta isomers of BHC, table IV, is essentially a delaying action similar to that reported by CORNMAN for colchicine (**11**) and D'AMATO for gamma benzene hexachloride (**12**). It is suggested that glucose may act for a time principally as a phosphate acceptor coincident with the stimulated fermentative reactions induced by the BHC (**4, 20**). High concentrations of BHC may quickly disorganize the system through which the glucose protection operates (**33**).

The effect of DNP at 10^{-4} M in subduing the C-tumor reaction induced by BHC at 5×10^{-4} M is a type of antagonism frequently reported (**5, 16**). SANTARATO (**39**) found that streptomycin in association with sulphapyridine in molar ratio of 1:2 decreased both the respiration and glycolysis of the system with which he worked. Further study is required to establish whether the association of DNP with BHC alters the course of tumor formation by some modification of respiration and fermentation.

A close relationship in point of time is noted between the altered phos-

phate metabolism in the roots shown in figure 4, and the lowered growth index in figure 2 for the treatment with BHC at 5.7×10^{-4} M. The accelerated release of inorganic P and the decrease in labile P occurred after 14 hours. Similarly, the irreversible effect of BHC as measured by the growth index in the transfer experiments and the altered phosphate metabolism after 14 hours are believed to be causally related. These results support the belief that BHC affects cellular function by inhibition of enzyme systems involved principally in reactions of synthesis. At the same time, the similarity between the effect of DNP and BHC in causing an accelerated release of inorganic P suggests initial activation of hydrolytic enzymes (**33**). The low values for labile P, though not a measure of ATP *per se*, are suggestive of accelerated hydrolysis of labile phosphate esters in the roots after treatment with DNP or BHC. McElroy (**33**) cites numerous examples in which inhibitors of cell division may cause the rapid breakdown, or prevent the formation, of certain energy-rich phosphate esters.

Summary

The effect of four purified isomers of benzene hexachloride and 22 additional compounds on the growth of seedling roots was determined by the use of a cell counting technique. The delta isomer is equal to or greater in phytotoxicity than the gamma isomer. The alpha isomer in high concentration inhibits growth and causes formation of a C-tumor in squash roots, but has neither effect on safflower roots. The implication of these data is discussed.

The inhibition of growth of squash roots by low concentrations of benzene hexachloride, sodium arsenate, DNP and iodoacetate is causally related to the inhibition of the number of non-vacuolated or meristematic cells between 24 and 48 hours.

Irreversible effects of the gamma isomer on root growth after 14 hours contact are indicated by data from transfer experiments.

A relationship in point of time was noted between growth inhibition and the phosphate metabolism in squash roots induced by high concentrations of gamma benzene hexachloride. This finding is discussed.

Among 22 compounds studied for their protective effects against concentrations of gamma and delta benzene hexachloride inhibiting growth, D-glucose was the most effective. In addition, 2,4-dinitrophenol antagonized the C-tumor reaction induced by gamma benzene hexachloride.

The alpha, beta, and gamma isomers of BHC were purified by the Department of Chemistry, University of Maryland, from crude materials furnished by the Penn Salt Company. The purified delta isomer was furnished by the California Spray Chemical Company. The writer gratefully acknowledges the valuable contributions of all cooperating individuals.

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FIBER PROPERTIES AND CARBOHYDRATE AND NITROGEN LEVELS OF COTTON PLANTS AS INFLUENCED BY MOISTURE SUPPLY AND FRUITFULNESS

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(WITH TWO FIGURES)

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As distinct from the cereal crops and the spring-flowering fruits, which are characterized by a reproductive climax, cotton is like the tomato and cucurbits in its indeterminate growth; but it is different in the minor extent to which fruit size is influenced by environment and fruitfulness. In a regional variety study (**3**), 16 varieties of cotton planted during each of three years at eight locations showed locational variations in the varietal means of only 8% in fiber length, 6% in fiber weight-per-inch and 9% in fiber strength. In terms of use value, however, such differences have high significance. The tendency toward uniformity in the bolls of cotton is accounted for by the extent to which young bolls are shed as the plant becomes heavily fruited. Within varieties and environments, the number of developing bolls per unit of vegetative weight (**11**) has been found to be remarkably uniform.

From California irrigation experiments, ADAMS *et al.* (**1**) record that reductions in water supply result generally in losses in staple length (usually within 1/32 inch), seed index (usually within 1.0 gram) and lint index (usually within 0.5 gram). Drought has been observed to decrease the length of cotton lint (**2, 5, 18**); with this effect, an increase in fiber strength was usually found. BERKLEY *et al.* (**4**), as well as others, have associated the increased strength of fibers with a narrowing of the angle of the cellulose crystallites (x-ray angles).

Increased nitrogen supply has been found to result in some increase in length of fiber (**7, 16, 19**). On the other hand, little effect upon length was found by STURKIE (**18**), whereas, in the presence of inadequate supplies of some other elements, BROWN (**6**) reported length reductions to result from nitrogenous fertilizers. As has been the general experience with other plants, WADLEIGH (**19**) found increased nitrogen supply to result in lower carbohydrate accumulations. He associated as causal the plus and minus changes in carbohydrate or nitrogen with the changes in boll properties and in fiber length that corresponded in sign.

CROWTHER (**7**) and NELSON (**16**) have shown phosphate to increase boll size but not to alter fiber properties. Nelson also found that potash additions which greatly increased yield also increased fiber length and weight per inch; the x-ray angles were increased and the strength of fiber and yarn decreased. YOUNGE (**20**) has shown sulphur deficiency that reduced boll production had little effect on fiber properties.

Variation in moisture supply is perhaps the major cause of variation in cotton production in the United States. Second, in this regard, is the damage to the cotton crop caused by attacks of bud, boll, and leaf destroying insects and diseases. Disease and insect attacks alter the number of bolls produced and the leaf area and weight of the vegetative parts of the plant. Changes in relative fruitfulness are a general result of the cotton plant's development from the first boll to advanced fruiting.

This paper gives the results from a series of experiments in which the major experimental variables were fruitfulness and moisture supply. The effects of variation in these two factors were measured in terms of boll and fiber properties and in terms of associated responses in the carbohydrate and nitrogen accumulations within the plant. Response of the two variables in Texas are compared with results in California, and inter-varietal correlations between carbohydrates and fiber properties are reported. The influence of relative fruitfulness on fiber properties has been mentioned previously (**8**). Only in the case of nitrogen supply (**19**) has the intermediate metabolism of the plant been studied in relation to boll development and fiber length.

Methods

Except as noted, the experiments were of factorial design arranged to yield data for two levels of fruitfulness at each of two moisture levels. During the summers of 1944 and 1945, the plants were grown at College Station, Texas, and during 1946 and 1947 at Shafter, California. In 1947 plants at the higher moisture level were deflorated (maintained with but two bolls per plant). Additional data on moisture supply, but not on fruitfulness, are included from experiments at Temple, Texas, in 1944 and at Shafter in 1948. Several of the experiments included additional treatments and amplifications that are not pertinent to the present subject.

The chemical analyses were made on oven-dried tissues (75° C) ground to pass an 80-mesh screen by methods heretofore described (**12, 14**). All data are expressed as percentages of fresh weight. At the time of the 1944 work there was a concurrent interest in the susceptibility of cotton plants to Phymatotricum root rot. For this reason the root bark was analyzed. In 1945 and later, the middle third of the main stalk was always used as the principal tissue; but in some instances, the leaves from the middle third of the main stalk and the entire roots, as lifted with a shovel, were analyzed as well.

Results

The primary data are presented in four summary tables each calculated on the basis of factorial analysis, *i.e.*, the means of the two-bolls per plant treatment in wet and dry plots versus full-load plants in wet and dry plots and the means of full-load and two-boll plants in irrigated plots versus those in drier plots except in two experiments (Temple, 1944 and Shafter, 1947) when there was no defloration in the dry plots. Tables I and II re-

port, respectively, the percentage changes in the chemical constituents and in the fiber properties resulting from partial defloration. Tables III and IV report the corresponding changes resulting from low water supply.

Effects of partial defloration

Chemical changes.—The chemical data that apply to plants whose fruiting was limited to two bolls per plants (one boll in the instance of undersize plants and three bolls in the instance of large plants) are summarized in table I. With one exception, partial defloration resulted in a reduced moisture content of all tissues sampled. This effect is possibly attributable to the displacement of moisture within the cells by the extra accumulations of sugars, starches, proteins, etc.

TABLE I

EFFECT OF PARTIAL DEFLORATION ON ACCUMULATION OF CARBOHYDRATES AND NITROGEN IN VARIOUS TISSUES. DATA EXPRESSED AS PERCENTAGE CHANGE (FRESH WEIGHT BASIS) FROM NORMALLY FRUITED PLANTS.

	College Station		Shafter		
	1944	1945	1946	1947	
	Stoneville 2B	5 varieties	Acala p18	Acala p18	Stoneville 2B
Moisture					
Leaves			− 1	− 1*	− 1
Stems—middle third		− 3	− 2	− 4	0
Roots	− 7**		− 4		
Carbohydrates					
Leaves					
Hexose			15	1	0
Sucrose			0	21	19*
Total sugars			15	17	14*
Starch			22	5	29*
Total			19	9	20**
Stems—middle third					
Hexose		− 2	− 7	−28	26
Sucrose		34**	4	45*	15
Total sugars		5	− 5	18	18
Starch		107**	81	91	53*
Total		45**	20	38	31**
Roots					
Hexose	0		−12		
Sucrose	37**		− 5		
Total sugars	25**		− 6		
Starch	121**		68		
Total	60**		30		
Nitrogen					
Leaves					
Soluble N			− 6	14	1
Protein N			16	− 2	2
Stems					
Soluble N			0	17	0
Protein N			21	17	7
Roots					
Soluble N	18**		25		
Protein N			0		

*Significant at the 5% level.
**Significant at the 1% level.

TABLE II

EFFECT OF PARTIAL DEFLORATION ON BOLL AND FIBER PROPERTIES. DATA EXPRESSED AS PERCENTAGE CHANGE FROM NORMALLY FRUITED PLANTS.

	College Station		Shafter		
	1944	1945	1946	1947	
	Stoneville 2B	5 varieties	Acala p18	Acala p18	Stoneville 2B
Boll period—days	2**		− 1		
Seed cotton per boll	5**	16**	− 1	− 4	8*
Seed index	13**	8**	6	10	6
Lint index	4	7*	5	8	1
% Lint	− 7*	− 1	− 1	− 6	− 3
Fiber					
Length					
Upper half	2	3	3	3	3*
Mean	3	4	4	3	4**
Weight per inch	− 2	4	0	1	5*
Mature fibers	0	2	3	5	7*
Tensile strength	5**	3	− 1	− 5*	1
X-ray angle	− 5**				
Yarn					
Strength					
22s	7	7			
36s	4	8			
50s	7	5			
Combined	6**	7**			
Yarn-appearance grade	3	6			
Neps in card web	−11	−23			
Seed					
% oil	− 9**		− 6		
% NH_3	5**		1		

*Significant at the 5% level.
**Significant at the 1% level.

In each of the four summers in which boll setting was limited to two bolls per plant substantial increases were found in the concentrations of starch (5 to 121%) and in total carbohydrates (9 to 60%) in the tissues. Sucrose, with a minor exception, increased (−5 to 45%) as starch increased. In 1945, when five varieties of cotton were included in the tests, instances were found of substantial and significant differences between varieties in carbohydrate levels.

The two-boll plants, with few exceptions, accumulated more nitrogen in leaf, stem, and root tissues than did the full-load plants. This result is to have been expected since it is usual by late summer for 60 to 70% of the total nitrogen of the tops of cotton plants to be located in the fruiting structures. The gains were in protein N in some cases and in soluble N in others. Partial defloration has been shown (10) to increase, also, the nitrogen content of cotton lint.

Boll and fiber properties.—In 1944 there was a small but highly significant lengthening of the boll periods (days from flowering to first separation of carpel walls) of the plants limited to two bolls; possibly because of the poor fruiting of the control plants, this lengthening of boll period was not found in 1946. In 1946 the DDT preparation used for insect control

was not effective and as a consequence the control plants were sparsely fruited. With all varieties other than Acala p18, the limitation of fruiting caused an increase in weight of seed cotton per boll. In all years and varieties, the seed index (weight of 100 seeds) and the lint index (lint on 100 seeds) were increased. The foregoing responses indicate that partial defloration gave the bolls on the two-boll plants a nutritional advantage. The reduced percentages of lint are due to seed index having been increased more than lint index.

Partial defruiting reduced the percentage of oil in the seed whereas the percentage of NH_3 was increased. Calculations from the data show that in terms of oil and protein per seed (1944) there was a gain of 1.8% in oil and 16.5% in protein which indicates that with extra nitrogen in the boll, protein synthesis is favored over oil synthesis.

In all experiments partial defloration increased fiber length but only within the range of about 3%. The gains both in upper-half mean length and in mean length were consistent. Fiber weight per inch was increased significantly by 5% in one test; when the results in all tests are averaged, the gain was about 2%. Increases in the percentage of mature fibers (those with wall thickness greater than lumen diameter) occurred in four of the five trials, the average gain amounted to about 3%.

In the five varieties used in 1945, the tensile strength of the fibers was increased in three trials by partial defloration, but in both trials with Acala p18 the strength was decreased. The x-ray angles were measured only in 1944. In this test, the angle was narrowed by 5% which corresponded with a 5% gain in fiber strength. Yarn strength was measured in two of the tests; there was a gain of 6% in 1944 and 7% in 1945 and both increases were significant.

Effects of drouth

The data on the influence of drouth on carbohydrate and nitrogen accumulation are summarized in table III, and those on fiber properties are summarized in table IV. The extent of effects of drouth on the carbohydrate and nitrogen levels and on boll and fiber properties are related throughout the series of tests to the severity of the water deficiency. For this reason growth will be reviewed before considering the data of these tables.

Growth.—For the years 1944, 1945, and 1946, an index to the growth reductions caused by moisture deficiency is afforded by the weights of the samples collected for laboratory analyses. A series of detailed plant measurements were made in conjunction with the experiments of 1947 and 1948. In 1944 at Temple, Texas, laboratory samples were collected at five dates between July 17 and August 20. The average weight of root bark of plants without irrigation over the entire sampling period was reduced by an average of 31% relative to the plants irrigated; at the last sampling the reduction was much greater. At College Station, during the same dry summer,

TABLE III

EFFECT OF DROUTH ON ACCUMULATION OF CARBOHYDRATE AND NITROGEN IN VARIOUS TISSUES. DATA EXPRESSED AS PERCENTAGE CHANGE (FRESH WEIGHT BASIS) FROM IRRIGATED PLANTS.

	Temple	College Station		Shafter		
	1944	1944	1945	1946	1947	
	Stoneville 2B	Stoneville 2B	5 varieties	Acala p18	Acala p18	Stoneville 2B
Moisture						
Leaves				− 9	− 2**	− 3
Stems—middle third			− 2	− 13**	− 5**	− 1
Roots	− 6**	− 5**		− 15		
Carbohydrates						
Leaves						
Hexose				39	− 8	1
Sucrose				− 50	1	−15
Total sugar				15	− 2	−10
Starch				− 20	6	5
Total				− 10	3	− 3
Stems—middle third						
Hexose			12**	98**	− 9	58
Sucrose			16	500**	22	−26
Total sugar			13*	131**	12	− 3
Starch			4	479*	115**	31
Total			8	213**	31	8
Roots						
Hexose	19**	40**		106		
Sucrose	24*	12**		136		
Total sugars	22**	21**		123		
Starch	91**	32**		352		
Total	42**	26**		224		
Nitrogen						
Leaves						
Soluble N				75	2	1
Protein N				17	0	5
Stems						
Soluble N				100*	37	−12
Protein N				50*	7	− 7
Roots						
Soluble N	42**	21**		38		
Protein N				70		

*Significant at the 5% level.
**Significant at the 1% level.

the samples were collected three weeks after the first afternoon wilting in the unirrigated plots. At that time there was only a minor reduction in the weight of root barks from the irrigated plots, but the daily wilting of the unirrigated plants continued after this sampling; and by late August, the irrigated plants were twice as large as the unirrigated.

At College Station in 1945 the laboratory analyses were made of the middle third of the main stems. There were five varieties and five sampling dates. The averages of all of these values show that lack of irrigation reduced plant height by 9% and the weight of stems by 16%. Several July and August rains materially lessened the contrast between the irrigated and unirrigated plants. At Shafter in 1946, the dry plants were not irrigated

TABLE IV

INFLUENCE OF DROUTH ON VARIOUS BOLL AND FIBER PROPERTIES. DATA EXPRESSED AS PERCENTAGE CHANGE FROM IRRIGATED PLANTS.

	Temple	College Station		Shafter			
	1944	1944	1945	1946	1947		1948
	Stoneville 2B	Stoneville 2B	5 varieties	Acala p18	Acala p18	Stoneville 2B	Missdel × Acala
Bolls							
Boll period—days		− 6**		−28**			
Seed cotton per boll		−10**	− 9**	−35**	4	2	− 3
Seed index	− 8**	− 8**	− 7**	−32**	2	−1	−14**
Lint index	− 3	− 6**	− 9	−46**	6	1	−10**
% lint	− 3	0	2	− 3	3	0	2
Fiber							
Length							
Upper half	− 8*	− 3*	− 4*	−14*	1	3	− 7*
Mean	− 8*	− 4*	− 5*	−18*	0	2	−13*
Weight per inch	7	− 2	− 2	− 2*	3	4	− 7*
Mature fibers	5	− 2	− 2	−28*	2	1	− 7
Tensile strength	0	1	3	20**	1	−3	10*
X-ray angle	− 4*	− 4*					
Yarn							
Strength							
22s	− 8	− 4	1		5	1	
36s	− 9	− 7	0		5**	3**	
50s	−11	− 6	− 2		4**	0	
Combined	−10**	− 5**	0				
Yarn appearance grade	−14	−13**	−11**		1	3	
Neps in card web		68**	30		− 3	8	
Picker and card waste					−11*	5	
Seed							
% oil	− 6	− 2		−20*			
% NH_3	− 1	2		− 3			

*Significant at the 5% level.
**Significant at the 1% level.

after June 15; there were no rains. When first sampled on July 16 and 17, there were only minor differences in the weight of the weekly irrigated and unirrigated stem and root samples. On August 6 and 7, the weight of the middle third of 12 stems from the plants irrigated weekly was 193 grams and those from the unirrigated blocks 72 grams. The weights of 12 roots, as lifted with a shovel, were 372 and 203 grams, respectively.

The effects of drouth in 1947 and 1948 on plant growth was marked. In 1947 the low moisture supply caused no wilting but decreased the weight of leaves and stems of the Acala and Stoneville varieties by 53 and 50% respectively. In 1948 the Missdel × Acala plants with low moisture wilted severely between irrigations, and growth was reduced by 47%. In each instance the measurements were made at about the time the first bolls were opening.

CHEMICAL CHANGES.—The most pronounced wilting and the greatest curtailment in growth from moisture deficiency was obtained at Shafter in 1946. Both the irrigated and unirrigated plants were thinned to about two

feet to allow extra soil volume per plant. Relative to the irrigated cotton, table III, there were large reductions in the percentage of moisture in leaves (9%), stems (13%) and roots (15%).

The hexose sugars of the leaves in 1946 showed a slight increase but this increase failed to offset larger losses that occurred in the concentrations of sucrose and starch. Very different results were found in stem and root tissues. Total sugars were three times as high in the stems of the dry plants as in those irrigated weekly (3.26% and 0.98% respectively). There was an average of 1.68% of starch in the stems of the dry plants against 0.29% in irrigated stems. The effect of drouth on the carbohydrates of the roots was only slightly less pronounced. In this experiment drouth also caused gains of 38 to 100% in the amounts of soluble nitrogen of the leaves, stems, and roots. The moisture deficiency was less in the other years and effects of drouth on carbohydrate accumulations were also less. In the relatively dry summer of 1944, the gains in sugars plus starch in root tissues were substantial at both Temple and College Station. In the rainy summer of 1945 at College Station, however, the unirrigated plants accumulated only a little more sugar and starch in their stems than the irrigated plants and the latter made only a little more growth. At Shafter in 1947, moisture deficiencies that reduced growth to about half, but which caused no wilting, did not result in significant differences in sugars or starch in the leaf tissues (means of six sample collections) of either Acala p18 or Stoneville 2B. In the stems, both varieties showed gains in starch of substantial magnitude.

The effect of drouth on the nitrogen content of leaves and stems was more often positive than negative and in 1946 the gain was large.

Boll and fiber properties.—With a few minor exceptions, drouth resulted in losses in seed cotton per boll and in reduced seed and lint indexes, table IV. These reductions were 8 to 10% in both 1944 and 1945, and 30 to 40% under the extreme conditions of the 1946 experiment. In the latter year, the bolls under dry treatment opened at an average age of 42 days compared with 59 days for the irrigated plants.

Under drouth there were reductions in fiber length, weight per inch and percentage of mature fibers. These reductions were most marked in years when the severity of wilting was most marked. In 1947 when there was no wilting, but a great reduction in plant growth, there were small gains, rather than losses, in the foregoing properties. Yarn appearance was improved substantially by drouth in those instances when the differences were significant. In each of these instances, the numbers of neps were also increased. It was evident that the reduced fiber length with drouth improved yarn appearance more than it was injured by neps.

Fiber tensile strength under the severe moisture deficiency of 1946 showed a significant gain of 20%. In 1948, when there was also much wilting, there was a significant 10% gain. In Texas, the water deficiencies of 1944 at College Station and at Temple had little effect on fiber tensile strength. In the two years when there were significant increases in tensile

strength, it was accompanied by significant decreases in fiber length, uniformity ratio, weight per inch, and percentage of mature fibers. In two experiments of 1944 (Temple and College Station), the x-ray angles were measured and found to have been significantly decreased by drouth. In the tests of 1944 at Temple and College Station, yarn strength was significantly decreased by drouth. In the five varieties in 1945 there was no effect on yarn strength. In 1947, water limitation resulted in increased yarn strength of both Acala and Stoneville varieties. The years when yarn strength decreased were years when drouth shortened the fibers but had little effect upon fiber strength.

Carbohydrate levels and fiber properties of cotton in Texas and California

Substantial differences were found both in the moisture and carbohydrate levels of the stem samples, table V, and in the fiber properties, table VI, of plants grown in Texas and in California. College Station has a higher relative humidity than Shafter (in the late afternoon, 75% compared with 35%). The two locations have similar maximum temperatures, but College Station has a higher minimum temperature (71 compared with 61). College Station has occasional cloudy days; the Shafter sky varies between

TABLE V

CARBOHYDRATES IN NORMALLY FRUITED AND IRRIGATED PLANTS IN TEXAS AND CALIFORNIA. PERCENTAGES ON FRESH WEIGHT BASIS.

Year	Location, variety and tissue	Moisture	Carbohydrates			
			Hexose	Sucrose	Starch	Total
1944	Temple					
	Root bark, Stoneville 2B	75.2	1.01	1.70	1.11	3.82
	College Station					
	Root bark, Stoneville 2B	75.7	0.92	1.75	1.56	4.25
1945	College Station					
	Stems					
	Arkansas Rowden B4	67.2	1.40	.35	1.19	2.94
	Delta and Pine Land 14	66.2	1.65	.49	1.09	3.23
	Hi-Bred	67.2	1.64	.24	1.45	3.33
	Stoneville 2B	67.0	1.96	.33	1.27	3.55
	Oklahoma Triumph 92-1-1	65.9	1.69	.22	1.65	3.56
1946	Shafter					
	Acala p18					
	Leaves	78.6	.14	.09	.36	0.60
	Stems	76.8	.85	.08	.27	1.20
	Large roots	72.0	.49	.45	.68	1.62
1947	Shafter					
	Acala p18					
	Leaves	77.0	.13	.29	.44	0.85
	Stems	75.0	.46	.76	.40	1.61
	Stoneville 2B					
	Leaves	76.8	.12	.37	.41	0.91
	Stems	72.1	.36	.94	.77	2.08

TABLE VI

BOLL AND FIBER PROPERTIES OF NORMALLY FRUITED PLANTS IN IRRIGATED PLOTS IN TEXAS AND CALIFORNIA.

Year	Location and variety	Seed cotton per boll	Seed index	Lint index	Fiber length		Wt. per in.	Mature fibers	Tensile strength
					Upper half	Mean			
		gm.	*gm./100*	*gm./100*	*in.*	*in.*	*μg.*	*%*	*1000 lbs. sq. in.*
1944	Temple, Texas								
	Stoneville 2B	....	11.4	6.0	0.98	0.76	4.1	78	88
1944	College Station, Texas								
	Stoneville 2B	5.8	12.2	6.2	1.04	.81	4.2	76	88
1945	College Station, Texas								
	Arkansas Rowden B4	6.6	12.3	7.0	1.07	.90	4.3	85	82
	Oklahoma Triumph 92	5.5	11.5	6.3	1.02	.83	4.5	81	71
	Delta and Pine Land 14	5.4	10.7	6.8	1.11	.90	4.5	88	77
	Hi-Bred	6.5	10.7	7.4	0.85	.71	5.8	86	79
	Stoneville 2B	5.9	12.3	6.7	1.10	.88	4.1	83	81
1946	Shafter, California								
	Acala p18	8.4	15.7	8.8	1.10	.92	4.2	84	74
1947	Shafter, California								
	Acala p18	7.2	14.1	9.8	1.04	.79	5.9	76	67
	Stoneville 2B	6.6	14.0	8.7	.98	.74	3.8	74	76

clear and hazy. The College Station experiments were on Lufkin fine sandy loam with a claypan subsoil. In a companion experiment on this land there was little or no response to nitrogen, phosphate or potash fertilizers. The Shafter experiments were on Delano sandy loam which is without marked profile characteristics; the subsoil is texturally much like the surface. Due to high NO_3 content of the irrigation water (25 pounds N per acre foot), there was no response at Shafter to nitrogen fertilizers, nor was there a response to either phosphate or potash.

Notwithstanding the higher humidity at College Station, the stems of Stoneville and the other four varieties grown in 1945 were much lower in their moisture percentages than was the Stoneville variety at Shafter in 1947. The stems of the Stoneville variety at College Station contained 67.0% moisture and at Shafter 72.1%. At College Station the stems contained more total sugar (2.28% compared with 1.3%) on a fresh weight basis and also more starch (1.27% compared with 0.77%). For samples collected from plants of similar planting date and age, the College Station plants (113 days) were 95 cm. tall and the Shafter plants (115 days) were 94 cm. tall. The middle third of the stems were heavier at College Station than at Shafter, but these weights are not indicative of growth relations because the Shafter plants developed many more vegetative branches (due to cooler nights) than the College Station plants. That the higher carbohydrate levels at College Station were not due to differences in fruitfulness is indicated by the fact that at 113 and 115 days the two-boll plants at College Station contained 4.9% total carbohydrates and the two-boll plants at Shafter, 2.92%.

In 1944 and 1945, the Stoneville variety at College Station contained 5.8 and 5.9 grams of seed cotton per boll and at Shafter in 1947, 6.6 grams; the differences in seed index and lint index were greater. The Stoneville fibers at College Station were longer, weighed more per inch, and were stronger than at Shafter; but the yarn strength was the same at the two locations. As calculated from seed cotton per boll, seed index, and lint index, there were 31.5 seeds per boll at College Station and 29.0 at Shafter; thus there were many more fibers per seed at Shafter since the lint index was much higher there and the length of fibers and weight per inch were less.

TABLE VII

TOTAL CARBOHYDRATES (SUGARS AND STARCH) IN THE STEMS OF FIVE VARIETIES OF COTTON SAMPLED AT FIVE DATES. VALUES EXPRESSED AS PERCENTAGES OF FRESH WEIGHT. COLLEGE STATION, 1945.

Variety	Dates	Treatments				Mean
		Wet full load	Dry full load	Wet two-boll	Dry two-boll	
	7–17	2.58	2.91	2.86	2.50	2.71
	7–25	3.20	3.48	2.81	3.15	3.16
	8–1	3.55	4.14	4.12	5.93	4.44
	8–8	3.58	3.50	6.11	6.63	4.96
	8–20	3.69	4.00	8.23	7.95	5.97
	Mean	3.32	3.61	4.83	5.23	4.25
Arkansas Rowden B-4		2.94	3.21	3.95	4.47	3.64
Delta and Pine Land 14		3.23	3.31	4.23	5.24	4.00
Hi-Bred		3.33	3.56	4.78	4.65	4.08
Stoneville 2B		3.55	4.00	4.96	5.83	4.58
Oklahoma Triumph 92-1-1		3.56	3.96	6.22	5.97	4.93
	Mean	3.32	3.61	4.83	5.23	4.25

	DF	*Mean square*	*LSD between any two means*
Treatment	3	21.43	
Dry vs. Wet	1	2.98	
Full vs. Two-boll	1	61.23	
Interactions	1	0.08	
Varieties	4	5.14	
Dates	4	35.17	
Variety × treatment	12	0.65	
Variety × date	16	0.42	
Treatment × date	12	7.05	
Error (T × D × V)	48	0.37	
Total	99	3.46	
Treatment	3	21.43*	0.35
Variety	4	5.14**	0.39
Error (T × D × V)	48	0.37	
Treatment	3	21.43*	1.64
Date	4	35.17*	1.83
Error (T × D)	12	7.05	
Variety	4	5.14**	0.39
Date	4	35.17**	0.39
Error (T × D × V)	48	0.37	

*Significant at the 5% level.
**Significant at the 1% level.

TABLE VIII

CARBOHYDRATES, PLANT GROWTH, BOLL, AND FIBER PROPERTIES OF FIVE VARIETIES OF COTTON AT COLLEGE STATION IN 1945. ALL VALUES ARE MEANS OF FOUR TREATMENTS SHOWN IN TABLE VII.

Variety	Carbohydrates			Stems		Seed cotton per boll	Seed index	Lint index	Length upper half	Fiber wt.	Mature fibers	Tensile strength
	Hexose	Sucrose	Starch	Height	Middle third							
	%	%	%	*cm.*	*gm.*	*gm.*	*gm./100*	*gm./100*	*in.*	μg.	%	*M lbs.*
Arkansas Rowden	1.53	0.46	1.64	106	7.3	6.4	12.3	6.9	1.06	4.4	84	84
Delta and Pine Land	1.63	.42	1.95	89	6.2	5.3	10.6	6.6	1.08	4.6	87	80
Hi-Bred	1.83	.35	1.89	86	6.2	6.4	10.7	7.8	0.84	5.8	87	79
Stoneville	2.01	.38	2.19	84	5.6	6.0	12.5	6.6	1.10	4.1	83	83
Oklahoma Triumph	1.73	.44	2.76	87	6.5	5.6	11.3	6.4	1.03	4.7	79	73
LSD, 5% level	.18	.16	.30	2.9	.49	.34	.28	.07	.004	.17	2.7	2.0

CARBOHYDRATE LEVELS AND FIBER PROPERTIES OF FIVE VARIETIES OF COTTON

Five varieties were included in the 1945 experiment to determine if varieties might be characterized by their carbohydrate levels as well as by plant type, vigor of growth, and fiber properties and to compare the reactions of these varieties to partial defloration and drouth with the responses observed in Stoneville 2B in 1944. Only the middle thirds of the main stems were analyzed. As shown in table VII, there were significant differences between a number of the varieties in the accumulation of sugar plus starch but no significant difference in the reactions of the five varieties (item 7 of the mean squares) to the four treatments.

In all treatments, Arkansas Rowden had the lowest carbohydrate and either Oklahoma Triumph or Stoneville 2B was highest. No interaction was found between varieties and dates of sampling. A significant interaction between treatment and date of sampling was found, *i.e.*, with the advance of the summer, plants in the two-boll treatment gained more in carbohydrate level than those in the full-load treatment. In all varieties and treatments there were increases in carbohydrate level with the advance of the summer. The mean concentrations of hexose, sucrose and starch for all treatments and dates in each of the five varieties are reported in table VIII. There were significant differences between some of the varieties in hexose sugars and in starch but not in sucrose; Stoneville had the highest hexose content and Triumph the highest starch. The ratio of starch to hexose was extra high in the latter variety.

Plant heights, mean weights of the middle third of the stems and the mean values for the fiber properties are reported in table VIII. In all of these characters there were significant differences between some of the

TABLE IX

INTER-VARIETAL SIMPLE CORRELATIONS* FOR SUGARS, STARCH, AND TOTAL CARBOHYDRATES WITH VARIOUS PLANT, BOLL, AND FIBER PROPERTIES. COLLEGE STATION, 1945.

Characters	Full-load plants			Two-boll plants		
	Sugars	Starch	Carbohydrates	Sugars	Starch	Carbohydrates
Plant						
Height	−0.83	−0.37	−0.78	−0.39	−.61	−0.65
Stem weight	− .82	− .17	.56	− .44	−.47	− .54
Boll						
Seed cotton per boll	− .66	− .20	− .54	.28	−.48	− .34
Seed index	− .28	.07	− .13	.07	−.18	− .13
Lint index	− .44	− .48	− .59	.04	−.52	− .44
Fiber						
Fiber length	− .01	− .18	− .12	− .25	.18	.07
Fiber weight	− .24	− .01	− .16	.05	−.27	.25
Mature fibers	− .46	− .80	− .81	.01	−.69	− .59
Tensile strength	− .08	− .60	− .44	.20	−.64	− .48

*Required for significance: 5% level 0.63, 1% level 0.76.

varieties. Carbohydrate levels in a selected tissue can at best be only a balance between such diverse processes as rates of photosynthesis, translocation, and utilization; all of which are undoubtedly influenced by a variety of factors. For this reason, it had not occurred to us that there might be inter-varietal correlations of carbohydrate levels with either growth or fiber properties. Some of the inter-relations apparent in the tabulated data, however, prompted the calculation of the simple correlation coefficients reported in table IX. The differences in carbohydrate levels between the wet and dry treatments being relatively small, there seemed to be some justification for combining these treatments in one array for the full load values and in one for the two-boll values. The most salient feature brought out by table IX is that all significant correlation coefficients had negative signs, both in the full load and in the two-boll groups. These negative correlations show that between varieties, differences in rate of carbohydrate use rather than differences in level of carbohydrate supply dominated the developmental responses.

The values for total sugars and total carbohydrate gave higher correlations with plant height, stem weight, and boll size than did starch; but starch gave higher values with mature fibers and fiber strength. Between varieties, neither starch nor sugar content showed significant relations with fiber length or fiber weight. When account is taken of the high carbohydrate levels in the two-boll plants, it seems surprising that the rate of use could have made enough of an inroad into the carbohydrate supply for negative correlations to exist with any of the plant or boll properties. It will be observed in table IX that as a group, the two-boll coefficients tended to be less negative than the full-boll coefficients. The sugar and starch relations under the two treatments appear to be somewhat different. In the coefficients for sugars, the full load plants are more negative than the two-boll in eight of the nine comparisons. On the other hand for starch, the reverse is found; the two-boll coefficients are more negative in seven out of the nine comparisons.

A number of relations are brought out in figure 1. The narrow range in carbohydrate levels between the five varieties in the full load series becomes a wide range in the two-boll series; this spread is two or three times as great in the latter. The segregation of the points for irrigated and non-irrigated plants (actually two groups of values) that is notable for height and weight in the full-load plants disappears in the two-boll plants. But even without the segregation, the coefficient for carbohydrate with height was significant. In an earlier paper (**9**), it was shown that removing the floral buds throughout a summer doubled the ratio of roots to tops. It appears here that the enlarged root systems of the two-boll plants enabled them to get moisture that was not within the reach of the full load plants. Attention was called earlier (**12**) to an observation by J. W. Neally that early determinant strains of cotton in the Mississippi Delta did not withstand drought nearly as well as later strains. This implies that a greater root development was associated with the greater vegetation development

of the less determinant cottons. The tendency in the full load plants toward separate grouping of the irrigated and non-irrigated plants shown in vegetative characters is not evident in the scatter diagrams for fiber strength.

Some features of the foregoing tabulations suggest that the carbohydrate supply of the varieties low in carbohydrates did not restrict their growth and fiber development. Evidence, however, that carbohydrate levels were

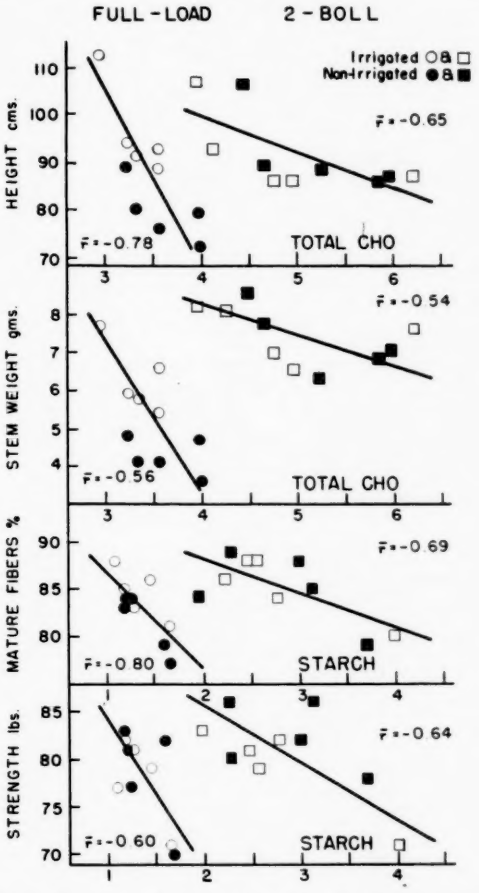

FIG. 1. Five-variety scatter diagrams, 1945. Top: Total carbohydrate against plant height and stem weight. Bottom: Starch against fiber maturity and strength. The results for the full load and two-boll plants are shown separately; those for irrigated and non-irrigated are combined. Carbohydrate and plant height and weight data are the means of five samples. Fiber maturity and weight data are the means of three pickings.

involved and that these did dampen growth expressions, and probably lessened the correlation coefficients, is provided by the rather consistent improvement in boll and fiber properties in the two-boll compared with the full-load treatments (table II). Since within varieties, growth and fiber properties were improved by the greater carbohydrate supply in the two-boll plants, it seemed possible that a variety low in carbohydrates such as Rowden or Delta and Pine Land would show greater improvement in the two-boll treatment than Stoneville or Triumph. Tabulations prepared to examine this point failed to support the idea. Instead, the tissues that were most responsive, and the extent of the response, appeared as further varietal characteristics. Delta and Pine Land, a small boll variety, had the second to lowest hexose and starch; and yet it showed the smallest increases under the two-boll treatment in height, seed cotton per boll, seed index, lint index and fiber length. Stoneville, with a high carbohydrate level, showed the greatest or second greatest response in height, stem weight, seed cotton per boll, lint index, fiber length, and mature fibers but the least response in fiber strength. Hi-Bred, a large-boll, small-seed, coarse-fiber, short-staple variety of intermediate carbohydrate level made the greatest gains of any in stem weight, seed cotton per boll, seed index, and fiber weight per inch.

Discussion

The two variables used in the foregoing experiments, partial defloration and limited water supply, were chosen because both would increase carbohydrate levels but at the same time with a distinction: Defloration imposes no limitation on carbohydrate use in vegetative expansion and in the remaining bolls whereas drouth does. In cotton (**13**), drouth limits growth more than it does photosynthesis. On the basis of findings with a number of other plants by other investigators, low nitrogen supply might also have been selected as a means of carbohydrate increase through growth limitation. For a satisfactory low nitrogen experiment under field conditions, it would be necessary to select soil that was without other complicating deficiencies. Also, under high light intensity (**12**) an increase in carbohydrate is not always found in cotton when nitrogen is limiting.

Water limitation increased sugar and starch accumulation in the stems and roots but usually not in the leaves. In contrast to the two-boll treatment, drouth decreased growth, boll size, seed index, lint index, fiber length, fiber weight per inch, and fiber maturity. Drouth decreased the percentage of oil in the seed and, in two out of three experiments, the percentage of NH_3 as well. The 1947 experiments with Acala p18 and Stoneville 2B at Shafter presented some exceptions. Irrigating on one side of the rows every two weeks with no wilting compared with irrigating both sides every week resulted in 50% reductions in plant growth. The dry treatment reduced the phototropic responses. As viewed toward the late afternoon sun, the leaves of the dry plants were more nearly horizontal and reflected more light to the eye than did the more heavily irrigated plants. All fiber properties,

other than strength of Acala fibers, were improved slightly in 1947 by insufficient water. These 1947 results with Acala and Stoneville varieties suggest that in the absence of wilting, the advantages of the higher carbohydrate levels on boll and fiber development may more than offset the disadvantages of reduced moisture supply. In bolls of these two varieties, 13 days old, the percentage of moisture was reduced 2 and 1% in the drouth treatment and 1 and 0% in the two-boll treatment. These samples (a total of 72 bolls for each treatment and variety) were collected during mid-morning and mid-afternoon on six days. The moisture reductions in the young bolls were only about half as great in both treatments as those found in the leaves at the same hours.

The strength of fibers tended to be improved in both the two-boll and drouth treatments with an increase in concentration of carbohydrate. Since fiber strength represents a formative effect in the deposition of cellulose which is reflected in the x-ray pattern, rather than a growth effect, the factors determining strength may exert their influence along different paths from those influencing the growth of the plant and its bolls and fibers. The factorial analyses in table II show that the two-boll treatment resulted in significant gains in the strength of Stoneville fibers in 1944, the strength of fibers of the five varieties in 1945, and a small gain in Stoneville fiber strength in 1947. Losses in fiber strength in the two-boll treatment were found in both the 1946 and 1947 experiments with the Acala p18 variety (the 1947 analyses were not factorial). Drouth resulted in large and significant gains in the fiber strength of Acala p18 in 1946 and Missdel × Acala in 1948; in the remaining drouth tests the changes were mostly upward but lacked statistical significance. Stoneville stems contained only 45% as much carbohydrate at Shafter in 1947 as at College Station in 1945. Possibly associated with this lower carbohydrate content, the Stoneville fibers in California had a round-bundle fiber strength of 76 whereas in three trials in Texas the strength was 88, 88, and 81.

The response of the variety Acala p18, to the two-boll treatment may be somewhat different from that of the other varieties. In support of this idea, it may be noted that in both tests with Acala p18 (in California only) there was a loss in seed cotton per boll under the two-boll treatment whereas the other varieties showed significant gains (table II). Exclusive of the Acala p18 variety and two additional minor exceptions, there was a positive association in the factorial summaries between carbohydrate level and tensile strength irrespective of whether the gains in carbohydrate resulted from the two-boll or drouth treatment.

As a means of examining this relationship between carbohydrate content and fiber strength apart from the averages of the factorial summaries, the 1944 and 1945 data are reassembled for more direct examination in table X. In this table, the treatment values for carbohydrates represent the mean of the three root-bark collections within a single 24-hour period in 1944 and the means of the five samples collected from each of the five varieties in

TABLE X

CARBOHYDRATE AND TENSILE-STRENGTH RELATIONS IN 1944 AND 1945 AT COLLEGE STATION. CARBOHYDRATE DATA EXPRESSED AS PERCENTAGES ON FRESH WEIGHT BASIS. FIBER STRENGTH IS IN THOUSANDS OF POUNDS PER SQUARE INCH.

	1944	1945					Means
	Stoneville	Rowden	Delta and Pine Land	Hi-Bred	Stoneville	Triumph	
Total Carbohydrates							
Wet, full load	4.25	2.94	3.23	3.33	3.55	3.56	3.48
Dry, full load	5.17	3.21	3.21	3.56	4.00	3.96	3.86
Wet, two-boll	6.58	3.95	3.95	4.78	4.96	6.22	5.12
Dry, two-boll	8.47	4.47	4.47	4.65	5.83	5.97	5.77
			LSD between any two means at 5% level				.77
Fiber Tensile Strength							
Wet, full load	88.5	82.0	77.3	79.7	80.7	70.7	79.8
Dry, full load	88.8	83.3	81.3	77.3	82.3	70.0	80.5
Wet, two-boll	92.5	83.0	81.0	79.7	81.7	71.3	81.5
Dry, two-boll	93.3	86.0	81.7	80.0	86.0	78.3	84.2
			LSD between any two means at 5% level				1.9

1945. The tensile strength values are the means of four pickings in 1944 and three in 1945. The 1946 data are not included because of the heavy loss of floral buds due to insects and the severity of the drouth treatment. There were no two-boll plants at Temple in 1944 and no two-boll plants in the dry plots at Shafter in 1947. The mean values from the six tests show highly significant increases in carbohydrate level and tensile strength due to drouth and defloration.

The x-ray angles were measured only in 1944. At College Station in 1944, the two-boll treatment decreased the x-ray angle by 5%; and there was a corresponding gain in fiber strength of 5%. Drouth also decreased the x-ray angle by 4%, and there was a 1% gain in fiber strength at College Station; but at Temple the reduced x-ray angle was not accompanied by an increase in fiber strength. For the four treatments at College Station in 1944, the x-ray angles were 29.17, 28.07, 27.86, and 26.80 degrees. The inter-relations in 1944 between carbohydrate and fiber strength are shown by scatter diagrams in figure 2. For significance at 0.05, a correlation coefficient of 0.95 is required for four pairs of values; a greater coefficient is found in the carbohydrate and x-ray angle relation and a smaller coefficient in the carbohydrate and fiber strength relation. An r value of -0.838 was found between x-ray angles and fiber strength.

The data of table X show a parallelism between fiber strength and carbohydrate levels in the supporting plants which may indicate a direct and causal relation but the possibility is not excluded that another physiological response to treatment is more direct even though associated with the carbohydrate response. Polar activities merit consideration in this connection. The data suggest a theory that the intensity of the polar forces of the lint cell, in an interaction with the polar glucose molecules, determines the

alignment of the cellulose crystallites in the cell walls of cotton during secondary thickening. MEYER's three dimensional illustration (**15**) of the positions of the polar OH groups of glucose and cellobiose precludes the possibility that the molecular axis passing through the 1 and 4 carbons can coincide with the polar axis. To account for the variable alignment of the crystallites from somewhat disorderly orientations, on the one hand, to orderly arrangements in the reversible spirals, on the other, it is helpful to believe that there are, within the lint cell, orienting forces that vary in intensity. The polar glucose or cellobiose molecules when moving about in the streaming protoplasm would tend to respond in orientation to the elec-

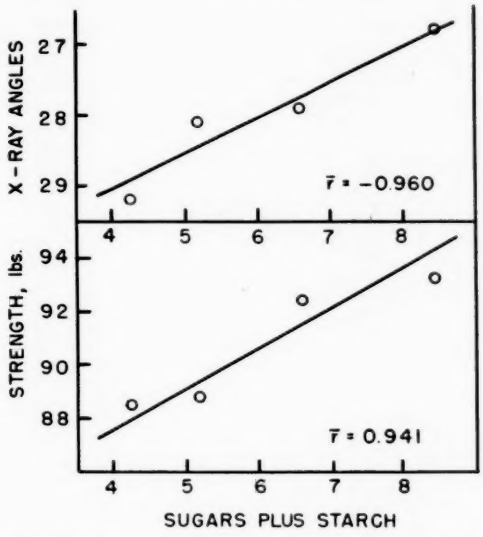

FIG. 2. Scatter diagrams of carbohydrate against x-ray angles and of carbohydrate against fiber strength, 1944 data. Note that the angle scale is from low to high and the strength scale from high to low. The carbohydrate data represent the means of three collections in one 24 hour period. The fiber data are the means of four pickings.

trical field of the lint cell. The orderliness of the orientation would thus be dependent upon the strength of the lint cell's electrical field. SPONSLER (**17**) believed that cellulose formation occurred at the interface between the cytoplasm and cell wall. Although he leaned heavily on the previous cellulose pattern as an orienting force, he called attention to the polarity of glucose and to the activity of the protoplasm in glucose utilization. It is reasonable to believe that cellulose formation takes place in the plasma film and also that the final orientation of the molecules occurs as they enter this film.

Meyer noted that there should be a large freedom of movement about the oxygen atom connecting the 1 and 4 carbons of two glucose units. As applied here, this freedom of movement when coupled with the varying

intensity of metabolic activity provides for variations from hour to hour and day to day in the angle of deposition. Also, a rotation of 180° about the molecular axis within a chain of glucose molecules would result in a change in the direction of their polar axes with respect to the cell axis, but it would not change the angle of the polar axes with respect of the cell axis. A reversal of direction without change of angle is characteristic of the spirals in the walls of the cotton fiber.

The inter-varietal comparisons of 1945 (tables VII, VIII, IX) are pertinent to a further discussion of the fiber strength relations. It will be recalled that between varieties the correlations of carbohydrate levels with fiber strength, as well as with plant, seed, and fiber growth, were negative rather than positive. Varieties that were low in carbohydrate, *i.e.*, used their carbohydrates most rapidly, had the greatest strength. And yet within each of these varieties, fiber strength increased with treatments that increased carbohydrate levels. As regards the path of influence on x-ray angles and fiber strength, it is indicated that both the potential of carbohydrate supply and rate of its use are involved.

Summary

The effects of restricting the number of bolls per cotton plant to two and limiting the water supply (on carbohydrate and nitrogen accumulation and on growth and fiber properties) were studied in a series of experiments at College Station, Texas, and Shafter, California. Both of the treatments increased carbohydrate levels and nitrogen in the plants, but there was the distinction that partial defloration did not limit the rate of carbohydrate utilization in vegetative growth and in the remaining bolls, whereas drouth did.

Under the two-boll treatment, increases occurred in plant growth, weight of seed cotton per boll, weight of 100 seeds, lint on 100 seeds, fiber length, fiber weight per inch, and fiber maturity. With extra nitrogen in the seed, protein synthesis proceeded at the expense of oil synthesis. For the most part, drouth reduced all of these characters; some exceptions were found, particularly in an experiment with the variety Acala p18, in which water limitation reduced plant growth by 50% but caused no wilting. The two treatments increased carbohydrate levels and usually increased fiber strength. Fiber strength represents a formative effect on the fiber cellulose rather than a growth effect. A few measurements indicated an inverse relation between carbohydrate and the orientation of the cellulose crystallites with respect to the fiber axis (x-ray angles).

In an experiment that included five varieties, those varieties showing the greatest growth and having the greatest fiber strength had the lowest carbohydrate levels in the stems. Within the varieties, however, the treatments that increased the carbohydrate levels (two-boll and drouth) also increased the fiber strength. The fiber strength was thus improved by the plant enzyme systems favoring rapid carbohydrate utilization and by the treatments that increased the carbohydrate supply.

The orientation of the polar glucose molecule as it enters the plasma film for polymerization into cellulose is apparently influenced by the intensity of the polar forces of the lint cell; and the intensity of this electrical field evidently varies both with the level of supply and rate of utilization of carbohydrate.

Carbohydrate levels were much lower in plants grown in California compared with plants in Texas, and fiber strength, length, and weight per inch were also lower; but in plants grown in California the weight of seed cotton per boll, weight per seed, and lint per seed were higher.

With the exception of the x-ray angles, the measurements of fiber and yarn properties were all made by the Cotton Branch of the Production and Marketing Administration. The measurements of x-ray angles and the analyses of cotton seed were made by the Division of Cotton and Other Fiber Crops and Diseases of the U. S. Department of Agriculture under the supervision, respectively of E. E. Berkley and W. H. Tharp.

The writers are obligated to C. B. Godbey and J. C. Gaines for advice on methods of statistical analysis.

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A CRITICAL STUDY OF THE COBALT CHLORIDE METHOD OF MEASURING TRANSPIRATION[1]

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(WITH THREE FIGURES)

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In recent years increasing awareness of the importance of water in our national economy has focused attention on the significance of forests in this important phase of conservation of our natural resources. Much has been written in the past 30 years concerning various aspects of the water relations of woody plants, yet available data concerning total water loss from trees and forests are inadequate. This inadequacy is apparent when it is realized that many of our present-day estimates are based on experiments conducted by von Höhnel prior to 1900 in which potted seedlings were used as test plants (**6, 7**). Such estimates have little value for appraising seasonal use of water by forests. The present study was undertaken to supply needed information concerning a measurement procedure which could be used with facility under field conditions to give reliable estimates of transpiration by forests of the Tennessee Valley.

Methods of measuring transpiration have been summarized from the literature (**5, 10**) and need not be described here. All of these methods are subject to criticism either on theoretical grounds or on the basis of operational difficulties. Most of them are impractical under field conditions, especially when applied to tree species. This is illustrated in the studies of Minckler (**11**) in which the water loss of 12 trees of five species was determined by an adaptation of the air-stream method. This procedure, one of the most acceptable under laboratory conditions, is quite cumbersome in application to field conditions.

The cobalt chloride procedure, first proposed by Stahl, has particular appeal for field studies since it requires no elaborate equipment and determinations can be made simply and quickly (**4**). Numerous studies of plant transpiration have been made with this procedure (**4, 8, 13**). Meyer (**9**) and Blaydes (**1, 2, 3**) have brought out a number of practical possibilities and limitations of the method and have emphasized that it does not measure absolute rates of transpiration that occur normally, but that the rates determined depend on certain abnormal conditions which may be imposed on the transpiring leaf surface by the cobalt chloride test paper. They considered that by careful standardization of the test paper the relative rates of

[1] This investigation was conducted in cooperation with the Tennessee Valley Authority, Division of Forestry Relations, while the first author was associated with the Department of Botany, University of Tennessee. It is published as Research Paper no. 1026, Journal Series, University of Arkansas, Botany and Bacteriology Department.

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water loss may be determined in a quantitative manner. This measure they have termed the standard water-vapor loss.

If the cobalt chloride method gives quantitative results, the values so obtained should have some definite relationship to absolute rates of water loss at the particular time of measurement. By establishing this relationship it should be possible to get at least a semi-quantitative measure of transpiration during the time of the test, and by repeated determinations to get improved estimates of water loss over an extended period. In view of the desirable simplicity of the method in field use, the present study was undertaken to evaluate critically the cobalt chloride procedure as a means of assaying plant transpiration. Experiments were designed for simultaneous measurements of water loss by weighing potted plants and by using cobalt chloride test papers.

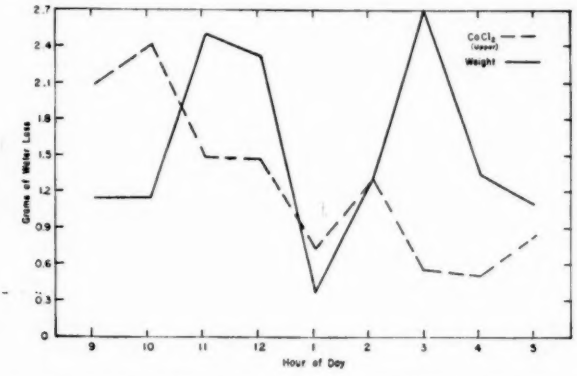

FIG. 1. Comparison of the cobalt chloride and gravimetric methods of measuring diurnal water loss of *Helianthus tuberosus* L. Data supplied by Dr. G. W. Blaydes.

Only one previous study is known in which the gravimetric and cobalt chloride methods of measuring transpiration have been compared. BLAYDES (**2**) determined hourly rates of water loss by *Helianthus tuberosus* L. simultaneously by the two methods. His results are summarized graphically in figure 1 by curves drawn through hourly observations. The weight curve is based on accumulated water loss during each hour. The cobalt chloride curve is based on averages of five determinations on different leaves during a small part of each hour. It is evident from the curves that transpiration measurement by the two methods gave poor agreement during most of the day. These results suggest that either the sampling by cobalt chloride tests was not extensive enough to determine a reliable average moisture loss or that comparable results cannot be obtained by this method and gravimetric determinations. Blaydes concludes that close correlation of the data obtained by the two methods should not be expected.

The present study was undertaken as an exhaustive comparison of the cobalt chloride and gravimetric methods of measuring transpiration. Ex-

periments were designed with particular attention to the requirements for adequate sampling with cobalt chloride tests and to statistical evaluation of the results obtained (**12**). The study was conducted in the greenhouse and under field conditions. This paper presents a brief description of the experiments and a discussion of the results to the point of conclusions on the limitations of the cobalt chloride technique as an index of transpiration.

Experimental procedures

Cobalt chloride paper was prepared by a method similar to that described by MEYER (**9**). Several circular filter papers 11 cm. in diameter (S. and S. no. 589, blue ribbon) were impregnated with a 3% solution of cobalt chloride slightly acidified with a few drops of hydrochloric acid. These were rolled gently on a squeegee board to remove excess solution and partially dried between sheets of porous paper in an oven at 50° C. When the edges of the paper began to turn blue, it was removed from the oven and drying was completed with an electric hand iron. Some of the paper circles prepared in this manner had to be discarded because they were impregnated unevenly. From the circles of acceptable quality, as judged by the uniformity of blue color in the dry paper, five square test sheets seven cm. on a side were cut. Calibration of these squares involved determining the gain in weight associated with the change from blue to a standard pink color. The initial blue color was achieved by drying in an oven at 60° C and cooling in a desiccator over anhydrous calcium chloride. The paper squares were placed in tared weighing bottles to minimize absorption of water during weighing. Subsequent exposure to moderately humid air in the laboratory resulted in a change to the pink color. The end point for this change was standardized by reference to a standard pink color sheet impregnated with 0.002% eosin. Final weighing in the tared weighing bottles gave the moisture absorption by the paper squares in changing from the initial blue color to the standard pink end point. The different test squares and replicate tests with these gave results of satisfactory uniformity. The average moisture absorption was 0.38 mg. per cm.^{2} of paper.

Cobalt chloride clips were made in the manner described by MEYER (**9**). Cellulose acetate film was cut into strips 1.2 cm. wide and 6.2 cm. long. Two disks of impregnated paper one cm. in diameter were placed on one side of the strip, one at each end. Each disk was held in place with Scotch tape in which a hole 7 mm. in diameter had been punched. Folding the strip at the middle made a clip which, when applied to a leaf, exposed circles of cobalt chloride paper 7 mm. in diameter to each surface of the leaf. In early phases of this study a strip of filter paper impregnated with 0.002% eosin was placed beside each disk of cobalt chloride paper to provide a standard pink end point. This practice was discontinued as it became apparent that experienced operators did not benefit by this refinement. COPELAND (**4**) notes a similar experience. The cobalt chloride clips used in transpiration tests were selected from the orginal group on the basis of uniformity of time required for the color change from blue to pink. Some clips were

discarded because their rates of change were abnormally fast or slow. The cellulose acetate strips were attached to leaves for transpiration tests with ordinary paper clips (fig. 2).

Transpiration standards were determined by the loss of weight of plants grown in cylindrical pots of soil that were paraffin-sealed against evaporation. The total tare of the potted plants did not exceed 1.5 kg. which permitted the use of triple beam balances sensitive to 0.1 gram for the gravimetric determinations. The unit time interval for measuring water loss was one hour, with half-hour and two-hour intervals in single experiments. Transpiration from the potted plants ranged from 0.4 to 24.7 grams per hour, determined as weight loss.

FIG. 2. A cobalt chloride clip applied to a leaf of a southern red oak tree, *Quercus falcata* Michx.

Species were represented in an experiment by two to five plants which were selected from a larger group available for this purpose. For the cobalt chloride tests leaves on each plant were numbered and subdivided into leaf positions. Leaves of white oak and black oak and tobacco were divided into six sampling areas, three on each side of the midvein. Geranium leaves were divided into five similar areas for sampling, and each of the seven or more leaflets of tomato leaves was considered a sampling position. Cobalt chloride tests were assigned at random to leaves and leaf positions of each test plant.

Preliminary cobalt chloride tests were made on either five or six plants of each species prior to their use in the experiments to establish the order of variability that might be expected between plants of a single species, leaves on a single plant, and positions on individual leaves. In these tests, positions on a single leaf showed less variation than that from leaf to leaf;

and it was determined that sampling could be randomly distributed among leaves and leaf positions. Compared with the variation resulting from this sampling, the plant to plant difference proved high for each species. Analysis of the data obtained in these preliminary experiments indicated the number of cobalt chloride tests necessary in a unit time interval to achieve a desired level of precision for each species. In this manner it was established for tobacco, tomato, and the two species of oaks that 16 tests per unit time interval were necessary to give an average with a standard error that was 30% of the mean. Two out of three averages so obtained could be expected to fall within 30% of the true mean. With careful planning a single operator in an hour can make 16 tests on each of two plants, or a total of 32 tests. To get an average with a standard error of 3% would require 1600 tests in an hour, which is, of course, entirely impractical. Preliminary tests with geranium plants indicated somewhat less variability, requiring only eight tests to obtain precision comparable to that for the other species used in the tests. These results indicate a high order of variability in tests with the cobalt chloride technique. Further refinements of the clip technique to reduce this variability should be possible, but efforts in that direction did not give promising results. Evidently the major source of variability is in the plants. The gravimetric method of measuring water loss properly integrates local variations in the rate of transpiration at various positions on the plant which are evident from results obtained with cobalt chloride clips.

Cobalt chloride tests on the lower surface of leaves gave color changes ranging from rapid at 35 seconds to slow at 20 minutes. The latter was considered the slowest color change practical to record. Water loss from the upper surface of leaves was not included in the determinations. Preliminary tests on all species used in these studies showed that the cobalt chloride rates for the upper surface were very slow, often requiring as long as 120 minutes for the color to change. Apparently the stomata on the upper surface in tobacco, geranium, and tomato contributed very little to transpiration compared with the lower surface. Stem losses cannot be assayed by the cobalt chloride technique. Since such losses are insignificant in the total, the small errors they introduce in transpiration calculations can be neglected. This point has been confirmed in the laboratory with geranium plants.

Results of cobalt chloride tests are obtained initially in units of time, since the measurement involves determining how much time is required for the paper to absorb sufficient moisture to change from blue to pink. Such time values have a reciprocal relationship to water loss inasmuch as quick changes in color of the paper indicate high rates of water loss and slow changes indicate low rates of water loss. In this study each time value was converted to water loss units—the reciprocal relationship. The conversion was facilitated by the preparation of a table that gives water loss in mg. per hour per cm.^{2} for cobalt chloride readings by intervals of tenths of minutes.

The formula used for converting time to water loss was $1/X \times 60$ min. $\times$ calibration factor = water loss, where X is time in minutes required for change in color of the cobalt chloride paper, and the calibration factor is the gain in weight in mg. of one square cm. of the cobalt chloride test paper in changing from blue to pink, 0.38 mg. in the present experiments. Water loss data so obtained are expressed in mg. per hour per cm.^{2}

Transpiration experiments with small potted plants

Five experiments were conducted with the small potted plants to compare transpiration measurements by the gravimetric and cobalt chloride methods. The first four of these were conducted in the greenhouse; the last was in the field. Each of the experiments involved from three to nine successive time intervals between 9 A.M. and 5 P.M. of a single day. Intervals were two hours in the first experiment, one hour in the second, and one half hour in the fourth. Each species used in an experiment was replicated with two to five plants. The potted plants were weighed at the beginning and end of each time interval to determine water loss gravimetrically. Throughout the intervening period cobalt chloride determinations were made by three to five operators experienced in the technique. Thus, each interval on each potted plant provided corresponding observations on a gravimetric and cobalt chloride basis. Upon completion of an experiment, leaf areas were measured with a planimeter. Transpiration was computed on a unit-leaf-area basis for both cobalt chloride and gravimetric observations. Rate of moisture loss was expressed in the common denomination of mg. per hour per cm.^{2} for comparison of the two methods.

In the first greenhouse experiment four plants each of tobacco (*Nicotiana tabacum* L.) and geranium (*Pelargonium zonale* Willd.) were tested during three successive two-hour intervals, as indicated in table I. Each cobalt chloride observation was based on 16 determinations for tobacco plants and eight for geranium plants—the numbers required to give comparable precision of 30% of the mean. The subsequent experiments, conducted in a manner similar to the first, were extended to include the three additional species: Marglobe tomato (*Lycopersicum esculentum* Mill.), seedling black oak (*Quercus velutina* Lam.) and seedling white oak (*Q. alba* L.). In the last experiment small potted white oak and black oak plants, balances, and other equipment were moved into the field to determine if the results obtained under field conditions were similar to those obtained in the greenhouse studies. Experimental details for all the tests and the results of the variance analyses are summarized in table I. A supplementary table presenting the actual water loss data for all tests has been prepared and is available for distribution on request.

Variations in moisture loss among replicate plants of the same species in the same experiment were surprisingly high, having statistical significance in 9 out of 12 gravimetric tests and 6 out of 12 cobalt chloride tests (table I). This variability between potted plants was characteristic of all species

TABLE I

SUMMARY OF EXPERIMENTS WITH THE COBALT CHLORIDE AND GRAVIMETRIC METHODS OF MEASURING TRANSPIRATION SHOWING THE ORDER OF VARIATION BETWEEN PLANTS IN EACH TEST. THE SIGNIFICANCE OF THE VARIATION BETWEEN REPLICATE PLANTS IS EXPRESSED AS NS—NO SIGNIFICANCE, S—SIGNIFICANCE, AND HS—HIGH SIGNIFICANCE.

Exp. no.	Species	Method of measurement	No. of intervals	No. of plants	Plant variation	Standard deviation
						mg./hr./cm.²
1	Geranium	$CoCl_2$	3	4	NS	5.3
		Gravimetric	3	4	S	0.6
	Tobacco	$CoCl_2$	3	4	NS	2.2
		Gravimetric	3	4	NS	1.0
2	Geranium	$CoCl_2$	6	2	S	2.3
		Gravimetric	6	2	S	0.7
	Tobacco	$CoCl_2$	6	2	S	3.0
		Gravimetric	6	2	S	1.2
	Tomato	$CoCl_2$	6	2	NS	1.1
		Gravimetric	6	2	NS	0.7
3	Geranium	$CoCl_2$	6	2	NS	3.1
		Gravimetric	6	2	S	0.7
	Black oak	$CoCl_2$	6	2	HS	0.5
		Gravimetric	6	2	NS	1.4
	White oak	$CoCl_2$	6	2	HS	0.6
		Gravimetric	6	2	HS	1.0
4	Tomato	$CoCl_2$	4	5	NS	0.6
		Gravimetric	4	5	S	1.0
	Black oak	$CoCl_2$	4	5	NS	0.3
		Gravimetric	4	5	HS	0.9
5	Black oak	$CoCl_2$	9	5	HS	1.4
		Gravimetric	9	5	HS	0.9
	White oak	$CoCl_2$	9	3	HS	1.2
		Gravimetric	9	3	S	1.4

used in these experiments. Standard deviation, which is the measure of variation in moisture loss unaccounted for in the experiments, was fairly stable in the gravimetric determinations, averaging one mg. per hour per cm.² for all tests. Standard deviations for the cobalt chloride determinations varied widely about an average of 1.8 mg. per hour per cm.² The gravimetric determinations had much less unexplained variation in these experiments.

Average rates of transpiration for each species by experiments are presented in table II. Geranium and tobacco had relatively high rates of water loss in comparison with other species tested. The lowest rates were shown by the two species of oaks and tomato occupied an intermediate position. In general, rates obtained with cobalt chloride clips were higher than those obtained gravimetrically.

The relationship of moisture loss determined by the two methods was examined statistically. The regression of gravimetric loss showed signifi-

TABLE II

AVERAGE RATES OF TRANSPIRATION FOR EACH SPECIES AND EXPERIMENT AS DETERMINED GRAVIMETRICALLY AND WITH COBALT CHLORIDE CLIPS.

Species	Experiment no.	Transpiration	
		$CoCl_2$	Gravimetric
		mg./hr./cm.²	
Geranium	1	11.2	9.2
	2	10.0	9.0
	3	18.5	12.3
Tobacco	1	12.4	6.7
	2	11.6	9.3
Tomato	2	9.1	6.5
	4	2.4	4.7
Black oak	3	6.3	6.1
	4	1.0	3.5
	5	5.6	5.0
White oak	3	2.3	3.4
	5	5.7	3.8

cant increase with cobalt chloride loss except for geranium in experiment one and tomato in experiment four. These differed significantly from the regressions for the other 10 tests. Gravimetric moisture loss from geranium increased 1.5 mg./hr./cm.² for each increase of 10 mg. determined by cobalt chloride. Corresponding trends for other species were 2.0 in 10 for tomato, 2.6 in 10 for tobacco, 4.5 in 10 for black oak, and 7.0 in 10 for white oak. No regression was found sufficiently reliable to estimate gravimetric moisture loss from plants on which losses were measured only with cobalt chloride because the levels of moisture loss show uncontrolled differences between plants, species, and experiments. Thus the use of cobalt chloride for quantitative estimation of standard gravimetric moisture loss was found impossible. Cobalt chloride may still serve as a qualitative index of relative transpiration. The relationship of the two methods appears to be well described by the significant correlation coefficients of 0.57 for the three herbaceous species combined, 0.53 for black oak and 0.58 for white oak.

The relation of the cobalt chloride and gravimetric methods of measuring transpiration can be characterized further by examination of the data for individual pots in the various experiments. Figure 3 presents six examples representative of the total of 38 graphs for individual test plants. These were selected from the experiment in which potted seedlings of white and black oaks were tested under field conditions. The curves for the gravimetric data are based on weight loss during each hourly interval. The corresponding points on the curves for cobalt chloride determinations are the averages of 16 tests on each potted plant.

These graphs illustrate the general relationships of the cobalt chloride and gravimetric determinations obtained in all the experiments. In some

instances good agreement between the results for the two methods prevails, as in the case of white oak number 1 and black oak number 1. On the other hand, in the same experiment the curves for black oak number 3 are quite dissimilar, and all of the graphs show certain points of dissimilarity. Under some conditions the two methods seem to measure the same variable,

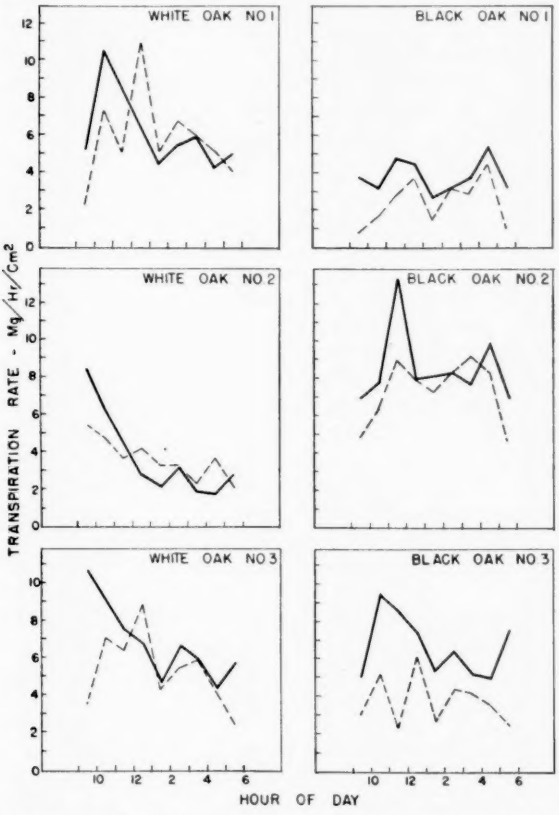

FIG. 3. Graphs obtained for representative potted plants showing relations of the cobalt chloride (solid line) and the gravimetric (broken line) methods of measuring transpiration.

but at other times they vary in an independent manner. In most of the graphs the calculated rate of water loss for the cobalt chloride determinations was somewhat higher than the corresponding gravimetric rate.

Variations in moisture loss between replicate plants are evident by either method of measurement. The curves of gravimetric determinations for

white oaks number 1 and number 3 and black oak number 3 are similar, while those for black oak number 1 and number 2 show little relationship to any others obtained. Examples of similarity and dissimilarity within the cobalt chloride data are evident also. These differences between replicate plants in an experiment have been described previously as statistically significant.

Discussion

The principal objective of this study was to determine if a cobalt chloride technique could serve to measure transpiration of plants quantitatively. Previous investigators (**1, 3, 9**) have considered that relative rates of moisture loss can be measured quantitatively by this method. A procedure which gives relative rates in a quantitative manner should give results which have some definite relationship to absolute rates of water loss from a particular plant unit. For purposes of this test transpiration was considered to be correctly measured by the loss of weight of potted plants, and the cobalt chloride measurements were characterized in relationship to this standard. Considerable effort was made to refine techniques to assure reliable observations with particular attention given to the sampling requirements for the cobalt chloride method.

The results of the tests show that on some occasions the cobalt chloride method gave water-loss values similar to those obtained gravimetrically. However, at other times, results by the two methods were very divergent. There is little chance that faulty techniques could account for the discrepancies obtained. The cobalt chloride method apparently measures some particular component of the transpiration process which can vary independently of the total moisture loss as measured gravimetrically. Just what is measured with the cobalt chloride procedure is uncertain, but it cannot be relied upon for transpiration measurement. Cobalt chloride may serve as a qualitative index of relative transpiration, but it is not possible to estimate the quantity of moisture loss from plants by cobalt chloride determinations alone. It is of interest to note that the field experiment fully supports the conclusions evident in the greenhouse experiments.

The experiments reported here have brought out the variability in the rates of moisture loss which occurs between plants of a single species and between leaves on one plant. Potted plants which appear quite similar may differ significantly in their transpiration rates as measured gravimetrically. Cobalt chloride tests on different leaves show a high order of variability. It is evident that the cobalt chloride method of measuring transpiration is not satisfactory for quantitative field studies. If transpiration of trees growing under natural conditions is to be determined with acceptable reliability another method must be used. Such a method should involve a minimum of heavy equipment and should be simple and rapid in its use. A method having these desirable characteristics and also proven reliability is not known at the present time.

Summary

The major objective of the present study was to determine if a cobalt chloride technique could be used to measure absolute rates of transpiration in a quantitative manner, with a view to possible application of the method in field studies. Gravimetric water loss from small potted plants was adopted as the standard of comparison for cobalt chloride determinations. Experiments were designed with particular attention to the requirements for adequate sampling with cobalt chloride tests and to statistical evaluation of the results obtained. Plants used in the tests were tobacco (*Nicotiana tabacum* L.), geranium (*Pelargonium zonale* Willd.), tomato (*Lycopersicum esculentum* Mill.), black oak (*Quercus velutina* Lam.), and white oak (*Quercus alba* L.).

The results of five transpiration experiments show clearly that quantitative data can be obtained with the cobalt chloride procedure, but these data show no consistent relationship with gravimetric observations. Good agreement between the two methods was obtained occasionally, but frequently the results were very divergent. The cobalt chloride technique apparently measures some unidentified determinant of the transpiration process which can vary independently of the total moisture loss as measured gravimetrically. Potted plants which appear quite similar may differ significantly in their transpiration rates as measured gravimetrically. Cobalt chloride tests on different leaves show a high order of variability.

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THE EFFECT OF LIGHT AND NITROGEN LEVELS ON GROWTH AND ALKALOID CONTENT OF YOUNG PLANTS OF *CINCHONA LEDGERIANA*

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(WITH ONE FIGURE)

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In Puerto Rico, seedlings of *Cinchona ledgeriana* Moens are usually grown in shaded nurseries for 12 to 18 months before transplanting to the field (**5**). The planting material resulting from this practice is usually weak and subject to disease. In Hawaii, TANADA (**4**) found that seedling plants of *Coffea arabica* L., which, like cinchona, are often grown in shade, grew better and were able to utilize nitrates better without shade. Conversely, GUISCAFRE and GÓMEZ (**2**) found the yield and growth of coffee in Puerto Rico to be better under one third to one half sunlight than under two thirds or full sunlight; but the coffee plants under full sunlight accumulated a greater amount of nitrogen than those in other treatments.

Experiments were undertaken to determine if stronger and more disease-resistant cinchona trees could be grown for field planting if the seedlings were grown under optimum light and nutrition conditions while in the nursery. Little or no published information is available on the effect of light and nitrogen and their interaction on the growth of cinchona. In 1946 an experiment was started at the Toro Negro cinchona plantation to determine the light and nitrogen requirements of cinchona plants during the nursery stage.

Materials and methods

The experimental treatments consisted of four levels of light and three levels of nitrogen in factorial combination. Sixteen nursery beds with shelters were constructed (fig. 1) on a level area of land from which all trees and brush had been cleared. The surrounding area was also cleared so that the light striking the experimental site was fairly uniform. The beds were $1 \times 3 \times 15$ feet, and the shelters over the beds were 18×6 feet with a height of six feet in front and four and one half feet in the rear. Each bed consisted of one of the four light treatments. These were spaced 12 feet apart in a Latin square arrangement four deep and four wide.

The light intensities less than full sunlight were obtained by differentially spacing two-inch laths on a frame over and around three sides of the beds. The north side or front of the beds was left open. The laths were spaced 0.7, 2.0, and 6.0 inches apart. A transparent glass substitute was tacked over all the shelters to prevent damage to the plants and leaching of the soil from the heavy rains. The full-light treatment was slightly less than maximum because of the glass substitute, but the light was reduced propor-

tionately in other light treatments. Light readings taken with a Weston photometer at plant level under the lath houses before the experiment was started and several times thereafter indicated that the light intensities were full sunlight and 68, 45, and 30% of full sunlight.

Four inches of rock were placed in the bottom of the beds, and the remaining eight inches were filled with a uniform mixture of light soil and sand which contained 66 p.p.m. of nitrate nitrogen. At the end of the experiment the soil in the check or low nitrogen plots contained an average of 15 p.p.m. Each bed was divided into three equal sections or subplots for the low, medium, and high nitrogen treatments by partitioning the beds with sheets of galvanized iron. The *Cinchona ledgeriana* seedlings used in the study were progeny of a single tree at Maricao, Puerto Rico. Seed were

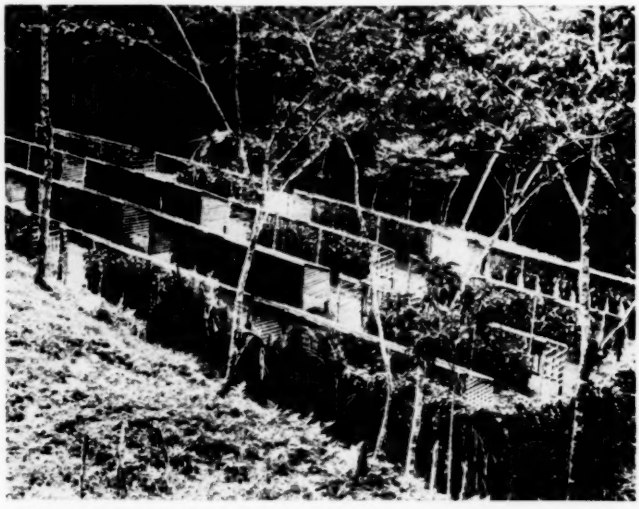

FIG. 1. General view of lath shelters constructed in a forest clearing.

planted at Toro Negro in September 1945, and were transplanted to intermediate nurseries in May and June 1946. While in the intermediate nursery, the plants were protected from direct sunshine and rain by a palm-leaf roof supported about three feet above the soil. On October 9 and 10, 1946, 40 uniform plants were selected and transplanted to each of the subplots which were randomized within each bed. At the time of setting the plants, all treatments were completely shaded from direct sunlight by placing palm leaves over the lath frames. When the plants had become established, the palm leaves were gradually removed until the plants were receiving the light intensity allowed by the spacing of the lath.

The nitrogen treatments were started immediately after the seedlings were transplanted. These treatments were applied by watering the soil in each plot once a week with three gallons of a nutrient solution containing

no nitrogen in the case of the low N treatment, 50 p.p.m. in the case of medium N treatment, and 200 p.p.m in the high N treatment. The nitrogen was supplied in the form of ammonium nitrate. All the nutrient solutions contained 40 p.p.m. K, 24 p.p.m. Mg, 11 p.p.m. P, 80 p.p.m. Ca, 3 p.p.m. Fe, 0.5 p.p.m. B, 0.5 p.p.m. Zn, and 0.1 p.p.m. Cu. All of the plots were watered, as needed, between applications of nutrient solution so that soil moisture was never a limiting factor.

Records on growth response were made at intervals throughout the experiment and growth measurements were made at the end of the experiment in November 1947, approximately one year from the time it was started. At that time the plants were crowding against the roofs of the shelter houses. Half of the plants from each plot were harvested and the growth data which included height, fresh, and dry weights were obtained. Each plant was weighed individually, and the roots, stem, and leaves were separated. The remaining plants were transplanted to the field for observation on growth and survival.

The total alkaloid content, quinine sulphate content of the roots and stem, and total nitrogen of the leaves and roots were determined by analyzing a composite of the plants in each plot. The method described by LOUSTALOT and PAGAN (**3**) was used for the determinations of total alkaloids and quinine. The A.O.A.C. method (**1**) was used to determine total nitrogen.

In October 1948 the shelters were rebuilt, the old soil was discarded, and new sand and soil were brought in. Essentially the same experiment was repeated except for minor changes in procedure because it was felt that in the first experiment the plants were so crowded against the roof of the shelters at the time of harvest that their growth response to the treatments might have been affected. Also it was felt that the control of light in the first experiment was not so complete as desired because the north side of the shelters was open. In the second experiment the front of the shelters was enclosed with laths spaced as required for each light treatment. Although the relative difference in light transmittance among the light treatments remained essentially the same as in the first experiment, the amount of reflected light striking the plants in the second experiment was at least one fourth less than it was in the first. The same procedures were used in applying nutrient solutions and water, harvesting, compositing, and analyzing the material.

The plants in the second experiment were harvested during October 1949. At this time they were not crowding the roof as in the previous experiment; and, therefore, the growth data obtained are considered to reflect the effects of the treatments more accurately than those from the first experiment.

Results and discussion

As a group, all of the plants grown in both experiments were the best that have been grown in any of the cinchona trials in Puerto Rico to date. Growth was uniform and good in all plots. Early in each experiment, after

the removal of the temporary shade used at time of transplanting, the plants growing in full sunlight and 68% of full sunlight showed yellowing of the leaves and reddening of the stems. Later these symptoms became less noticeable, and plants in all light treatments were more or less uniform. During the last two months of the experiments, symptoms of nitrogen deficiency developed in plants in the low- and medium-nitrogen plots of the beds with 30 and 45% of full sunlight. Plants in these plots had thin stems which tended to lodge in some cases. Plants of the low-nitrogen and low-light treatments had large thin leaves and in general were not as thrifty as those receiving medium nitrogen and light. Plots receiving high nitrogen were more thrifty than those receiving medium nitrogen and had a darker foliage color. Plants in the low-nitrogen, low-light plots shed more basal

TABLE I

TOTAL NITROGEN CONTENT OF LEAVES AND ROOTS (OVEN DRY BASIS) FROM CINCHONA PLANTS UNDER DIFFERENT LIGHT AND NITROGEN TREATMENTS.

Light treatment	Total Nitrogen					
	Leaves			Roots		
	Low N	Medium N	High N	Low N	Medium N	High N
	%	%	%	%	%	%
Full sunlight	2.43	2.39	2.70	1.00	0.94	1.07
68% sunlight	2.44	2.46	2.70	1.03	1.03	1.00
45% sunlight	2.61	2.75	2.91	1.09	1.06	1.08
30% sunlight	3.99	3.75	3.99	1.11	1.09	1.08
L.S.D., light treatments,		5% level	0.39			
		1% level	0.56			
L.S.D., nitrogen treatments,		5% level	0.13			
		1% level	0.17			

leaves than those in the high-nitrogen, low-light plots, which would indicate that even at the low level of light there was some deficiency of nitrogen.

In general, leaves of the plants grown in full sunlight and 68% full sunlight were of a lighter shade of green than those of the plants grown under lower light intensity, and they contained less nitrogen as is shown in table I. The trends in the analytical data from experiment 1 were essentially the same as those of experiment 2 given in table I. At all nitrogen levels there was a consistent tendency for the nitrogen content of the leaves to increase as the amount of light decreased. However, there was also a definite gradient in the intensity of green color from low- to high-nitrogen treatment as the amount of light increased. The trend in nitrogen content of the roots was similar but the differences between treatments were not statistically significant. In full sunlight, and almost to the same extent in 68% of full sunlight, the low-nitrogen plants exhibited symptoms of nitrogen deficiency. The leaves of these plants were yellow-green, smaller and narrower than

those of other treatments (high N). Shedding of basal leaves in these plots was extensive. The plants in medium-nitrogen treatments also exhibited these same symptoms but to a lesser degree. Seedlings receiving high-nitrogen treatment under full sunlight and 68% of full sunlight were the most thrifty, had better color, and were generally stronger than those receiving medium or low nitrogen.

TABLE II

AVERAGE HEIGHT AND DRY WEIGHT OF CINCHONA SEEDLINGS GROWN UNDER FOUR LEVELS OF LIGHT AND THREE NITROGEN LEVELS.

Light treatment	Nitrogen treatment	Height		Dry weight		Dry weight of roots	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
		in.	*in.*	*gm.*	*gm.*	*gm.*	*gm.*
Full sunlight	Low	39.9	27.5	67.5	33.0	11.6	6.0
	Medium	43.0	26.0	73.8	31.0	12.4	5.9
	High	47.1	29.4	83.5	41.8	13.1	7.0
	Average	43.4	27.6	74.9	35.2	12.4	6.3
68% sunlight	Low	39.8	27.3	60.0	30.5	9.9	4.9
	Medium	41.4	29.2	69.5	31.5	11.6	5.2
	High	43.8	28.9	85.0	35.5	12.2	6.0
	Average	41.7	28.5	71.5	32.5	11.2	5.4
45% sunlight	Low	45.0	31.3	62.5	30.0	10.2	4.6
	Medium	47.7	33.2	76.0	29.2	12.0	4.1
	High	45.0	33.9	75.8	33.8	10.6	5.3
	Average	45.9	32.5	71.4	31.0	10.9	4.5
30% sunlight	Low	41.1	28.3	52.8	18.5	7.2	2.4
	Medium	42.7	28.4	51.2	17.5	7.4	2.4
	High	45.5	31.7	78.0	21.5	10.2	3.0
	Average	43.1	29.5	60.7	19.2	8.3	2.6
Mean nitrogen values	Low	41.4	28.6	60.7	28.0	9.7	4.5
	Medium	43.7	28.9	67.6	27.3	10.9	4.4
	High	45.4	31.0	80.6	33.1	11.5	5.3
L.S.D., light treatment means							
5% level		N.S.*	N.S.	N.S.	5.49	2.18	0.83
1% level		N.S.	N.S.	N.S.	8.31	N.S.	1.26
L.S.D. nitrogen treatment means							
5% level		N.S.	N.S.	10.30	3.13	1.12	0.38
1% level		N.S.	N.S.	13.96	4.24	1.52	0.51

*Not significant.

The data for height and dry weight at the end of the two experiments are shown in table II. Each figure represents the average of four replications each containing 40 plants, except the figures for the average dry weight of experiment 1 which represent the average of 20 plants per replication. The other 20 plants were transplanted to the field.

In the second experiment, the plants grown under 30% of full sunlight had significantly less dry weight than those grown under the three higher light levels, but there was no statistically significant difference among the plants grown under higher light intensities. A similar trend is shown in the data for the first experiment, but the differences were not significant. The cinchona seedlings grown under high-nitrogen treatments in both experiments were significantly heavier than those grown with low or medium nitrogen, but there was no significant difference in dry weight between the plants grown in the low- and medium-nitrogen treatments. The statistical analysis of the growth data from the second experiment showed essentially the same trend as that found in the first experiment except that the differences were of higher significance probably due to the greater precision in the second experiment. The data for the average dry weight of the roots

TABLE III

TOTAL ALKALOIDS AND QUININE SULPHATE CONTENT OF ROOTS AND STEMS (OVEN DRY BASIS) OF YOUNG CINCHONA PLANTS GROWN UNDER DIFFERENT LIGHT AND NITROGEN TREATMENTS.

Light treatment	Total alkaloids						Quinine Sulphate		
	Roots			Stems			Roots		
	Low N	Medium N	High N	Low N	Medium N	High N	Low N	Medium N	High N
	%	%	%	%	%	%	%	%	%
Full sunlight	3.66	4.49	4.50	2.20	2.07	2.23	1.37	1.80	1.79
68% sunlight	0.72	4.15	3.58	2.21	2.47	2.43	1.05	1.60	1.29
45% sunlight	0.39	2.92	3.24	2.06	2.00	2.03	0	0.93	1.41
30% sunlight	0.22	0.42	0.31	2.14	2.21	2.30	0	0	0
L.S.D., light treatments,		5% level		0.46				5% level	0.31
		1% level		0.66				1% level	0.45
L.S.D., nitrogen treatments,		5% level		0.24				5% level	0.19
		1% level		0.32				1% level	0.25

of the plants show essentially the same results as the data for the average dry weight of the entire plant except that in the second experiment all values for light effect were statistically significant in descending order from full light to 30% of full sunlight. An analysis of variance of the height measurements from both experiments showed no statistically significant differences between either the light or nitrogen treatments. There was no statistically significant interaction between light and nitrogen in either experiment. The lower dry weight of the plants in the second experiment is attributed primarily to the fact that they were harvested a month earlier than the plants in the first experiment. Cool, clear days prevail during the fall at Toro Negro, and cinchona makes its best growth at that time of year.

The chemical analyses of the plants grown under the different light and nitrogen treatments of experiment 2 are presented in table III. The trends in the analytical data of experiment 1 were similar but not as pronounced. These data show that both light and nitrogen levels had a marked effect on

the total alkaloids and quinine content of the roots. Under higher levels of light and nitrogen the amounts of these constituents in the roots were larger. Although the per cent. of total alkaloids and quinine increased as either light or nitrogen increased, the greatest amount was formed when both factors were high. It is interesting to note that there was no quinine and very little total alkaloids formed in the roots of the plants grown under 30% of full sunlight regardless of the nitrogen level, but considerable amounts of these constituents were formed when light was high and nitrogen was low. There was no marked or consistent effect of the treatments on the total alkaloid and quinine content of the stems. Only a trace of quinine was found in the stems. The differences in alkaloid content of the stems were not significant.

The results of these experiments indicate that cinchona seedlings can be grown more or less satisfactorily over a rather wide range of nitrogen supply and light intensity, but the best growth was made when both light and nitrogen levels were high. The growth as measured by dry weight was significantly better at the three higher light levels than it was at the lowest light intensity, particularly when the nitrogen level was also high. Apparently cinchona can make good growth without shade if plenty of nitrogen is available.

The fact that total alkaloids and quinine increased significantly in the roots of the plants as light and nitrogen supply was increased indicates that both of these factors are necessary for the formation of alkaloids and quinine, but apparently light is the more important factor because no quinine and very little total alkaloid was formed in the roots at the lowest light intensity regardless of nitrogen treatment. On the other hand, considerable amounts of these constituents were formed in the roots of plants grown under high light intensity even when the level of nitrogen was low. The highest amount of quinine (1.8%) found in the roots of plants grown in full sunlight and with medium and high nitrogen supply is considered above average for cinchona plants of this age.

The rather uniform growth and survival of plants in all treatments may be due in part to the fact that water was applied uniformly, as needed, to all plots throughout the experimental period and also to the fact that the young seedlings were protected from the heavy rains which usually damage the plants.

Summary

Plants of *Cinchona ledgeriana* were grown under four light conditions, factorially combined with three levels of soil nitrogen, low, medium, and high. Early in the experiments the plants growing under 68% of full sunlight and full sunlight showed some yellowing of the leaves and reddening of the stems. Later these symptoms became less noticeable. Symptoms of nitrogen deficiency developed in the low- and medium-nitrogen plots as was evidenced by light colored leaves and stems. On the other hand, plants

receiving high nitrogen were thrifty and had a dark foliage color. Plants grown under 68% of full sunlight and full sunlight were able to utilize nitrogen better than plants grown under 30 or 45% of full sunlight.

Statistical analysis of height measurements made at the end of the experiment showed no significant differences between either the light or nitrogen treatments. Differences between light treatments, as measured by dry weight of the plants, were significant only between the low-light level and the three higher levels with no significance in dry weight between the three higher light intensities. Plants grown with high nitrogen supply were significantly larger than those with low or medium nitrogen supplies with no significant difference between the low- and medium-nitrogen treatments. The data for the average dry weight of the roots of the plants show essentially the same results as the data for the average dry weight of the entire plant. There was no statistically significant interaction between light and nitrogen on either height or dry weight.

The total alkaloid and quinine content of the roots increased as both light and nitrogen levels increased. The percentage of these constituents was highest when both light and nitrogen levels were high but light appeared to be the more important factor in the formation of cinchona alkaloids. There was no consistent or marked effect of the treatments on the quinine and total alkaloid content of the stems.

The authors wish to express their appreciation and thanks to Mr. Caleb Pagán for making the chemical determinations reported in these experiments.

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THE EFFECT OF CALCIUM ON THE ABSORPTION OF POTASSIUM BY BARLEY ROOTS[1]

ROY OVERSTREET, LOUIS JACOBSON AND RAYMOND HANDLEY

(WITH SIX FIGURES)

Received November 19, 1951

Introduction

In a recent investigation (**3**) the absorption of potassium by excised barley roots was studied. It was proposed that, as a working hypothesis, the absorption of ions depended upon the presence of metabolically produced binding compounds. According to this idea, the absorption of a cation may be expressed as: $M^+ + HR = MR + H^+$, where HR is the metabolically produced binding compound.

Studies of the absorption of potassium in the presence of H^+ and other monovalent cations appeared to be consistent with the above hypothesis; that is, H^+ caused what appeared to be a reversal of the absorption reaction and pronounced competitive effects were noted between potassium and the other cations. On the other hand, in accordance with the observations of VIETS (**5**), it was shown that the presence of calcium exerted a stimulating effect on potassium absorption. Since this calcium effect is in apparent conflict with the above hypothesis, a special study was made of the calcium-potassium relationship in absorption. The purpose of the present paper is to show that certain extensions of the theory of cation absorption appear necessary to explain adequately the behavior of calcium.

Experimental methods

The experimental material used was excised barley roots, cultured and prepared as described in the previous article (**3**). In general, the absorption experiments were performed using one gram of root tissue per liter of solution in order to minimize concentration changes during the course of the experiment. Usually 15 grams of roots were placed in 15 liters of solution. The root suspensions were continuously aerated throughout the experimental period, and with the exception of the isotopic exchange determinations, investigations were carried out at 26° C. Potassium was determined as the cobaltinitrite and calcium as the oxalate.

Results

In order to ascertain the rates of absorption of K^+ and Ca^{++} from pure salt solutions, absorption experiments were conducted using KCl solutions and $CaCl_2$ solutions. The observed absorption of the cations plotted as a

[1] This paper is based on work performed under contract no. AT-(11-1)-34, project 5, with the Atomic Energy Commission.

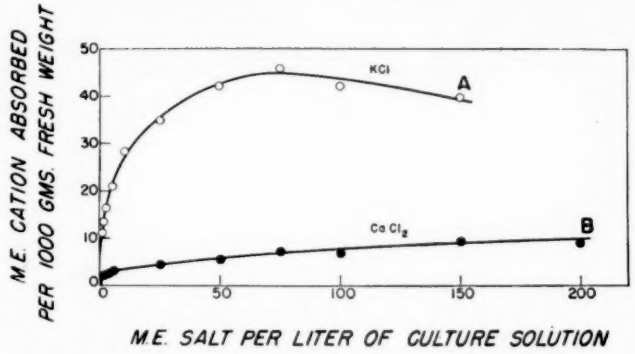

FIG. 1. A. Absorption by barley roots of potassium from a pure solution of KCl. B. Absorption by barley roots of calcium from a pure solution of $CaCl_2$.

function of salt concentration is presented in figure 1. The absorption period was three hours.

The effect of potassium on the absorption of calcium was determined by adding increasing amounts of KCl to 0.005 *N* $CaCl_2$ solutions and measuring the resulting calcium absorption (fig. 2). The reciprocal effect, the effect of calcium on potassium absorption, was determined in the following manner: A series of experiments was set up in which the calcium content was maintained constant for a given set and the potassium permitted to vary. Several levels of calcium concentration were employed. In this way,

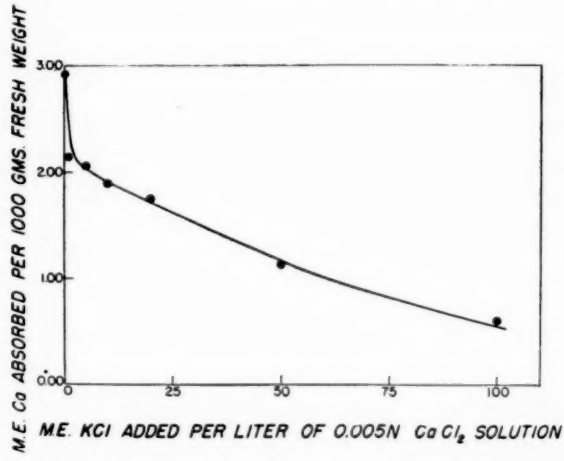

FIG. 2. Calcium absorption from 0.005 *N* $CaCl_2$ as affected by increasing concentrations of KCl.

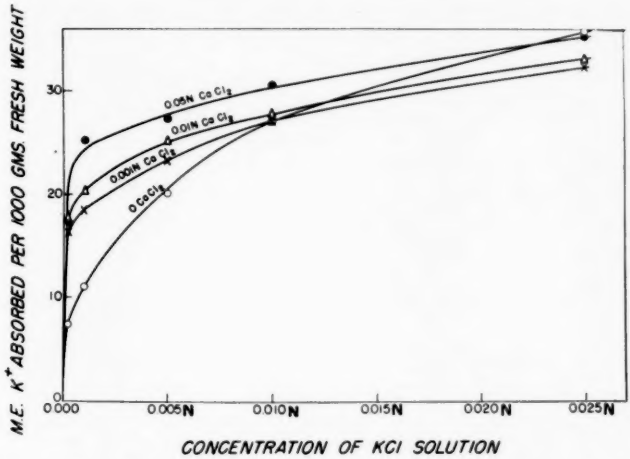

FIG. 3. Effect of different concentrations of $CaCl_2$ on the absorption of potassium from increasing concentrations of KCl.

the effect of the concentrations of both KCl and $CaCl_2$ on the absorption of potassium from the mixtures was observed. The results are summarized in figure 3. In this series of experiments, as well as in the preceding experiment, the absorption period was three hours.

Since the data of figure 3 indicated a marked dependency of the stimulating effect of calcium on the concentration of KCl, the following experiment was performed: A series of solutions was prepared in which the $CaCl_2$

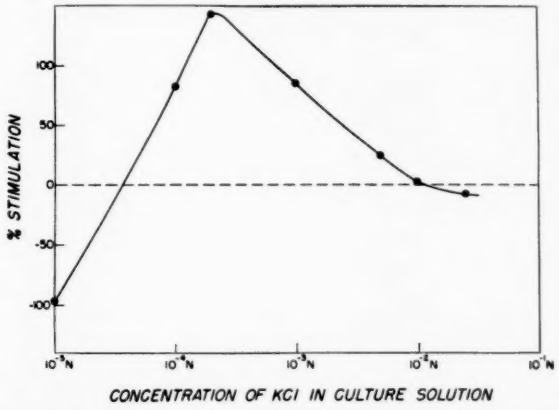

FIG. 4. Stimulating effect of 0.01 *N* $CaCl_2$ on the absorption of potassium from different concentrations of KCl.

content was kept constant at 0.01 N and the KCl content varied from 10^{-5} N to 2.5×10^{-2} N. The absorption from these solutions was compared with the absorption from solutions of pure KCl. From the data obtained, the quantity:

$$\% \text{ stimulation} = \frac{(\text{absorption from KCl} + \text{CaCl}_2) - (\text{absorption from KCl})}{(\text{absorption from KCl})} \times 100$$

was calculated for the various KCl concentrations. Therefore, in figure 4 a value of −100% indicates that $CaCl_2$ completely prevented absorption of potassium, a value of 0% stimulation indicates no effect of $CaCl_2$ on potassium absorption and +100% means the absorption of potassium was doubled by the presence of $CaCl_2$.

In another experiment, the effect of calcium on the uptake of potassium

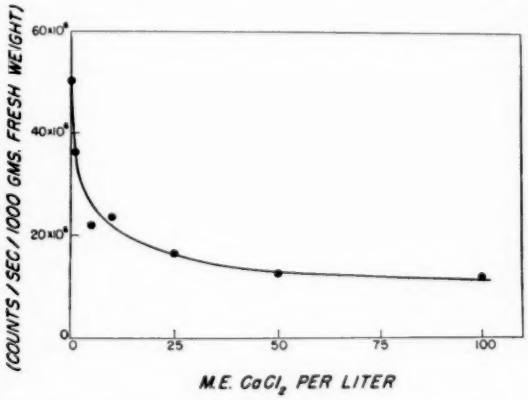

FIG. 5. The uptake of radio-potassium from a carrier-free solution of radio-potassium as affected by increasing concentrations of $CaCl_2$.

at the carrier-free level was determined. The potassium was prepared and isolated by a method previously described (**4**). The concentration of radio-potassium was calculated to be approximately 10^{-14} N. The solutions contained no inactive potassium other than that due to impurities in the water, reagents, etc. The uptake of radio-potassium at constant potassium concentration and varying $CaCl_2$ concentration is given in figure 5.

In order to determine the rate of isotopic exchange of potassium absorbed in the presence and absence of $CaCl_2$, the following experiment was conducted: The roots were divided into two batches and one batch was permitted to absorb potassium from 0.001 N KCl; the other batch absorbed potassium from 0.001 N KCl plus 0.01 N $CaCl_2$. In both cases, the KCl was labeled with radioactive potassium. After an absorption period of three hours, each batch of roots was removed from the solution, washed and centrifuged and further divided into two portions. One of these portions was then placed in 0.005 N KCl and the other in 0.005 N KCl plus 0.01 N

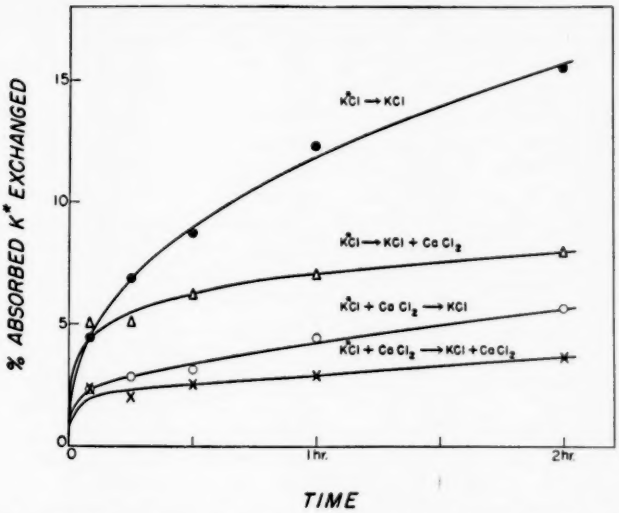

FIG. 6. Isotopic exchange of radio-potassium absorbed from 0.001 *N* K*Cl and from 0.001 *N* K*Cl + 0.01 *N* $CaCl_2$ and exchanged in solutions of 0.005 *N* KCl and 0.005 *N* KCl + 0.01 *N* $CaCl_2$. Absorptions carried out at 26° C and exchange reactions at 0° C.

$CaCl_2$. The other batch of roots was treated in the same manner. The exchanging solutions were kept at 0° C and the course of isotopic exchange followed by sampling the solutions for radioactivity at definite time intervals. The data for these experiments are given in figure 6.

Discussion

As may be seen from figure 1, the curves for absorption at increasing concentrations of potassium and calcium are markedly different. Aside from an initial rapid rise at low concentrations, the rate of calcium absorption is linear up to at least 0.200 *N* $CaCl_2$ concentration. In contrast to this, the absorption rate of potassium rises to a maximum at 0.080 *N* KCl and then falls. The character of the potassium absorption curve is markedly different from that observed in the low-salt barley roots of HOAGLAND and BROYER (**2**). In this latter type of material, the absorption rate rises to a maximum between 0.001 *N* and 0.005 *N* KCl and then remains virtually constant, *i.e.*, the absorption is independent of the concentration (**1**). The fall in absorption rate above 0.080 *N* KCl observed with the material used in these experiments deserves comment. It is doubtful that the decline is the result of osmotic effects since no corresponding behavior was noted with equivalent concentrations of $CaCl_2$. Thus far no adequate explanation for this decline has been found. Another significant difference between the up-

take of potassium and calcium is the considerably greater uptake of potassium.

The competitive effect of potassium on calcium uptake is clearly shown in figure 2. As the potassium concentration is increased, there is an initial rapid drop in the uptake of calcium and then a considerably slower falling off in the rate of uptake. This curve seems best interpreted as indicating that calcium is taken up both in the absorbed and the adsorbed form and potassium has a pronounced inhibiting effect on that fraction of the calcium which is absorbed and a considerably smaller inhibiting effect on the adsorbed fraction. In figure 1, the initial rapid rate of uptake also suggests that two fractions of calcium are involved.

However, the reciprocal effect, *i.e.*, the effect of calcium on potassium absorption, cannot be explained on so simple a basis. In this case, the absorption of potassium is markedly stimulated by the presence of calcium as is shown in figure 3. The data show that the effect is related to both the calcium and potassium concentrations in the external media. Even relatively small concentrations of calcium exert a stimulating effect, which increases as the calcium concentration increases. A study of figure 3 shows that for a given calcium concentration the effect diminishes as the KCl concentration increases. As the KCl concentration decreases, calcium has an increasingly stimulating effect down to a concentration of 2×10^{-4} *N* KCl (fig. 4). Below this value the stimulating effect diminishes and eventually the calcium depresses the absorption of potassium at very low concentrations of KCl.

The data of figure 5 indicate a depressing effect of calcium on radiopotassium uptake at extremely low potassium concentration (10^{-14} *N* plus reagent and glassware contamination). However, it should be noted that the depression in this case is not as extreme as at the higher concentration of 10^{-5} *N*. When roots are placed in a solution of potassium salt, both absorption and isotopic exchange occurs. At very low levels of potassium, *i.e.*, carrier-free radioactive solutions, isotopic exchange predominates. At higher levels, absorption predominates. For this reason, it is believed that figure 5 essentially shows the effect of calcium on exchange rather than on actual absorption.

In order to explain the interrelationships between calcium and potassium reported thus far, it is desirable to re-examine the mechanism proposed for ion absorption, namely, for potassium: $HR + K^+ = KR + H^+$. In this reaction, there are four rate determining factors, that is, the concentrations of the substances HR, K^+, KR and H^+. It can be expected that an increase in the amount of HR would lead to an increased rate of potassium absorption. Since HR is a metabolically produced substance, its amount would be dependent on temperature. The rate of absorption as a function of temperature has long been recognized and studied in root tissue (**2**). In the experiments described here, the temperature was constant and the root tissue metabolically uniform; hence, any variations in the quantity of HR must

be attributed to other factors. Likewise, an increase in K^+ should lead to an increased absorption rate. If the assumption is made that the concentration of K^+ at the reaction site is proportional to the external concentration, then the absorption rate should increase with increasing concentration of potassium in the external solution. That this is so is indicated by the data of figure 1 which show that the absorption rate rises with increasing potassium concentration until some other factor becomes limiting, possibly the amount of HR.

A decrease in the concentrations of KR and H^+ should also result in increased potassium absorption. In the experiments described, the concentration of the H^+ in the external solution was maintained at a constant value. However, considerable variations in H^+ concentration may occur at the site of the reaction. The level of KR is determined by the difference between its rate of formation and its rate of utilization. It is reasonable that in high potassium roots, the level of KR is higher than in low potassium roots; and hence the latter plants should absorb potassium more rapidly than the former. The more rapid absorption by low potassium roots has been observed experimentally (**2**).

On the basis of the foregoing considerations, the stimulating effect of calcium on potassium absorption would best be explained either as an enhancement of the rate of formation of HR or an increased rate of utilization of KR. In other words, calcium may act as a co-factor in the formation of HR or it may accelerate the removal of KR from the site of the absorption reaction by either chemical alteration or physical means. A corollary of the postulated absorption reaction is that exchange for absorbed potassium must occur through the mediation of KR. If the KR level is reduced through the agency of calcium, then the rate of the following isotopic exchange reaction should decrease: $K^*R + K^+ = KR + K^{*+}$. The data of figure 6 show that this is indeed the case. Had the calcium functioned as stimulating the production of HR, then the level of KR would have remained constant or increased and therefore we should not obtain a decrease in the exchange rate. On the basis of the isotopic exchange curves, it appears that calcium affects the level of KR rather than the production of HR.

To summarize, the interrelationships between calcium and potassium in absorption may be explained in terms of two simultaneous processes. The first of these is a competition between calcium and potassium for HR. The second is a depressing effect of calcium on the level of KR and a resulting stimulating effect on the absorption of potassium. Since potassium has a much greater affinity for HR than calcium (fig. 1), the competitive effect of calcium on potassium absorption becomes important only when the concentration of potassium is very low when compared to that of calcium. As the concentration of potassium increases, the competitive effect of calcium decreases and the stimulating effect becomes important. At still higher concentrations of potassium, unknown factors become limiting and the stimulating effect diminishes (fig. 4).

Summary

The interrelationships between potassium and calcium in absorption by barley roots have been investigated. It has been demonstrated that a given concentration of calcium exerts both a depressing and a stimulating effect on the absorption of potassium and that the effects are related to the concentration of potassium in the external media. The depressing effect has been interpreted as the result of a competition for a metabolically produced binding compound. In order to explain the stimulating effect, it is necessary to postulate that calcium functions as a cofactor in the utilization of the potassium complex produced during absorption.

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EFFECTS OF LIGHT ON ELONGATION AND BRANCHING IN PEA ROOTS

JOHN G. TORREY

(WITH THREE FIGURES)

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Introduction

The characteristic morphology of the branched tap-root system of an adult plant is determined by the number and location of lateral root meristems initiated by the primary root during the course of its development. Much interest has centered around the identification of the specific factors responsible for the initiation of the cellular divisions leading to the precise formation of lateral root primordia. It was shown by THIMANN (**16**) and others that auxin plays a role in the initiation of lateral roots in the primary root of such plants as the garden pea, *Pisum sativum*. Evidence from recent studies of isolated pea roots grown in sterile nutrient medium (**17**) indicated that some other factor or factors act together with auxin to control lateral root formation in this plant. The continued study of these unknown factors revealed some remarkable effects of light on root growth—effects which may contribute to our understanding of the processes controlling root growth.

Relatively few investigators have described direct effects of illumination on the growth of this usually subterranean organ of the plant body. Scattered reports in the literature including such early work as that by DARWIN (**2**) and others (**7**), demonstrated an inhibitory effect of white light on root elongation. SEGELITZ (**15**) reported that isolated corn roots grown in culture in the dark extend more rapidly than roots exposed to white light, an effect attributed to the production of auxin by roots in the light. ROBBINS and MANEVAL (**14**) reported that the growth of excised corn roots was favorably influenced by light, an effect also reported by WHITE (**20**) for the excised roots of wheat grown in sterile culture. NAUNDORF (**8**) showed that illumination causes increased auxin production in the roots of intact or seedless Helianthus plants, which results in a slight acceleration of root elongation. According to Naundorf, auxin stimulation of root elongation may occur when suboptimal concentrations of auxin are normally present in the root. Phototropic responses by roots, either positive or negative, are recorded for a number of different species of plants and attempts have been made (**8**) to explain these responses in terms of differential auxin production in the light and dark.

WHITE (**21**) has stated that light has no observable effect on the growth of tomato roots in a sterile nutrient medium. ROBBINS (**13**) reported a peculiar periodic formation of root hairs by isolated roots of Datura grown in

sterile culture. Root hair formation occurred during daylight hours and was accompanied by a reduced rate of root elongation.

There are some reports on the effect of light on root initiation. WENT (19) studied the effect of different wave lengths of light on the initiation of adventitious roots in etiolated pea stem cuttings. He found that illumination at any wave length decreases lateral root initiation by decreasing the effectiveness of the applied indoleacetic acid (IAA) on the root initiation process. Recently, GALSTON and HAND (5) suggested that light decreases the efficiency of IAA in the initiation of roots in stem cuttings by stimulating the IAA-oxidizing system present in the etiolated pea stem tissue. No reports have been found on the effect of light on lateral root initiation in roots.

In recent years, the study of plant responses to light has been found increasingly more useful in the analysis of physiological and biochemical phenomena. The experiments reported here on the effect of light on the growth of the roots of intact seedlings and isolated roots were done as part of a general study of the unknown factors controlling lateral root formation in the growing plant.

Materials and methods

In all experiments, seeds of the garden pea, *Pisum sativum* variety Alaska, were used. The seeds were surface-sterilized in 0.1% mercury bichloride, rinsed in sterile water, and germinated in the dark in Petri dishes at 25° C for 48 to 72 hours. In studies with intact plants, seeds were transferred aseptically to a 250-ml. Erlenmeyer flask containing 50 ml. of nutrient medium (1), consisting of mineral salts, vitamins, sucrose, and 0.5% agar. When plant parts were used, excision of the desired parts was done aseptically, and the parts were transferred to Petri dishes containing 20 ml. of nutrient medium. Cultures were maintained at 25° C either in the dark or in the light. Continuous illumination was provided in a chamber lighted from above by six 20-watt Mazda fluorescent tubes, three daylight tubes and three white tubes. Illumination of 100 foot-candles was obtained at the treatment surface as measured with a Weston photronic exposure meter. Other light sources used in short-term illumination experiments included tungsten incandescent lamps with and without gelatin filters of known transmission. These light sources are specified in detail for the experiments described. Unless otherwise noted, all manipulations of plant materials subsequent to the initial sterilizing procedure were done under indirect illumination from a 10-watt, frosted, incandescent lamp screened by a blue, gelatin filter with maximum transmission at approximately 4400 to 4800 Å. This choice of safe light was made on the basis of certain experiments described below.

Throughout these experiments the morphological response of lateral root formation is used as an index of physiological activity in the study of the factors responsible for initiating cellular divisions in the pericycle of the

primary root axis. The response *per se* is conveniently expressed as the average number of lateral roots initiated per root with 10 to 16 plants per treatment. It was found convenient in handling a large number of plants to count only lateral roots which were 1 mm. or more in length. Lateral root primordia less than 1 mm. in length are therefore not included in the counts. In order to eliminate a possible source of error arising from light effects due to inhibition of lateral root elongation, total counts were made on cleared roots from each type of light experiment to be described. Whole roots were cleared in 2.5% NaOH and stained with safranin, and all lateral roots, including root primordia as well as macroscopically visible laterals, were counted under a dissecting microscope. Such counts indicated that while the total number of lateral roots thus determined was consistently greater than counts made of visible laterals, the increases were closely comparable in all series regardless of treatment. Thus, a macroscopic count of lateral roots longer than 1 mm. is a reliable index of rooting response.

Experimental results

The respective roles of shoot and cotyledons in seedling root development

In early experiments it was noted that the root systems of intact pea seedlings grown on agar nutrient medium in the light and in the dark were quite different. Lateral root formation was markedly reduced in plants grown in the light. To establish the respective roles of the shoot and the

TABLE I

ROOT ELONGATION AND LATERAL ROOT FORMATION BY INTACT PEA SEEDLINGS AND ISOLATED ROOTS WITH ATTACHED PLANT PARTS GROWN IN STERILE NUTRIENT MEDIUM IN THE LIGHT AND IN THE DARK.

Series number	Type plant	Seventh day			Fourteenth day		
		Average shoot length	Average root length	Laterals per root	Average shoot length	Average root length	Laterals per root
		mm.	*mm.*		*mm.*	*mm.*	
			Dark				
1	Intact seedling	131	130	21.5	380	216	49.0
2	Root plus cotyledons	....	133	24.7	....	209	48.0
3	Root plus shoot	35	84	0.0	60	116	2.6
4	Root only	....	79	0.0	....	129	9.8
			Light				
5	Intact seedling	23	101	7.9	86	209	9.0
6	Root plus cotyledons	....	85	7.2	....	164	8.0
7	Root plus shoot	14	91	0.0	25	141	0.0
8	Root only	....	45	0.0	....	77	0.0

cotyledons in the formation of the root system of peas grown under each condition, experiments were conducted with plants in which the shoot, the cotyledons, or both cotyledons and shoot were excised following the 48 hour germination period. Plants were then transferred to agar nutrient medium in flasks and grown in the light or in the dark for two weeks. The results of one such experiment are presented in table I. It is apparent that, under these conditions, the shoot has no effect in the light or dark on the formation of lateral roots. The cotyledons, however, must be present for normal lateral root formation to occur, as has been shown by RIPPEL (**12**). Substances essential to the initiation of lateral root primordia are furnished by

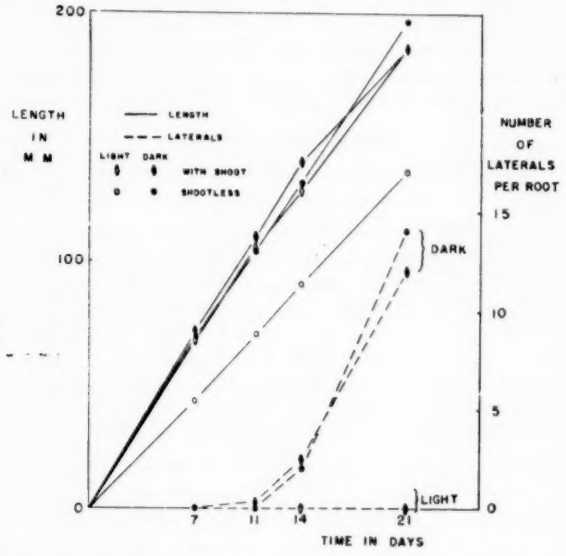

FIG. 1. Root elongation and lateral root formation in pea seedlings without cotyledons grown in white fluorescent light and in the dark.

the cotyledons and move into the primary root during the early stages of seedling growth. Attempts were made to substitute for the cotyledons by applying lanoline paste mixtures of IAA to the cotyledonary stumps. Such treatments had no effect on lateral root formation. In addition to auxin other substances apparently are provided by the cotyledons. Even in the presence of the cotyledons lateral root initiation is markedly inhibited by light.

To determine whether the shoot has any effect on lateral root formation during later stages of plant development, the cotyledons must be removed early during seedling ontogeny so as not to obscure any effect the shoot might exert. Pea seedlings without cotyledons, when cultured on agar nu-

trient medium in the dark, begin lateral root formation much later and at a slower rate than intact seedlings (Series 3, table I and fig. 1). In the dark, the presence or absence of the shoot has no effect on root initiation, as is shown in figure 1. Roots grown with or without the shoot produce 10 to 15 laterals per root in three weeks. Similar plants grown in the light do not form lateral roots at all. From the earlier experiments and the experiment summarized in figure 1, the following conclusions may be drawn: (a) the cotyledons are necessary for normal lateral root initiation in the intact pea seedling; (b) the shoot plays no measurable role in lateral root initiation whether or not the cotyledons are present; (c) in the absence of the cotyledons, lateral root initiation is delayed in plants grown in the dark, but will occur after a prolonged period of root elongation; and (d) lateral root initiation is inhibited in intact pea seedlings as well as cotyledonless plants by continuous white light.

Lateral root formation will be limited by the capacity of the roots to initiate lateral root primordia, a capacity which is determined by specific factors present within the primary root of the seedling. In the early stages of growth these factors are provided by the cotyledons; in later stages, the root appears to have the capacity to synthesize these factors or derive them from the nutrient medium. The experiments to follow were done with isolated roots, or excised roots with a single cotyledon attached, grown on agar nutrient medium in Petri dishes. The capacity of the isolated roots for the formation of lateral roots is initially limited by the substances already translocated into the root prior to the excision of the root tip; in roots with an attached cotyledon, the cotyledon provides a continuous supply of the factors necessary for lateral root formation.

The effect of light on roots with an attached cotyledon

A number of experiments were done to test the effect of duration of illumination on lateral root initiation. Seventy-two hours after germination,

TABLE II

EFFECT OF DURATION OF EXPOSURE TO LIGHT ON ROOT ELONGATION AND LATERAL ROOT FORMATION BY EXCISED PEA ROOTS WITH ATTACHED COTYLEDON.

Days of continuous light	Seventh day		Fourteenth day	
	Average root length	Laterals per root	Average root length	Laterals per root
	mm.		*mm.*	
0 (dark)	103	11.9	216	32.5
1	95	6.3	222	33.0
5	62	0.0	181	12.8
7	50	0.1	180	11.2
14	56	1.2	128	3.1
14 (red filter)	85	1.1	200	3.7

roots with a single attached cotyledon were transferred to nutrient medium in Petri dishes, two roots per dish, and placed under continuous illumination for a period of exposure varying from 1 to 14 days. After the illumination period, the roots were removed to a dark chamber until the total elapsed time was 14 days. In one series, a red filter (Corning Signal Red no. 2408 with transmission above 6100 Å) was interposed between the light source and the roots. The results, summarized in table II, indicate that during the period of illumination, lateral root initiation is almost completely inhibited (fig. 2 A). During the subsequent period of darkness, lateral root primordia

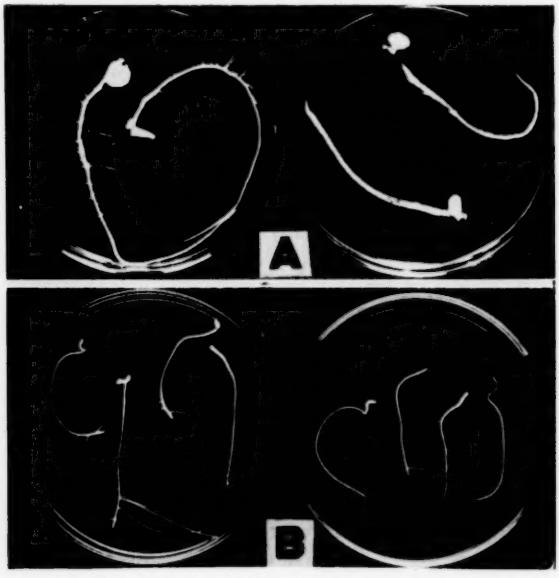

FIG. 2. A. Lateral root formation by isolated pea roots with attached cotyledon grown in sterile nutrient medium in the dark (left) and in continuous white fluorescent light (right). The photograph was made on the seventh day. B. Lateral root formation by decapitated pea roots grown in the dark (left) and in continuous white fluorescent light (right). The photograph was made one week after decapitation.

are initiated. It can be concluded that the light inactivates the factors necessary for lateral root initiation within the main axis of the root. Such light inactivation does not occur on exposing the cotyledon itself, either because of the dense nature of the cotyledon, or because the active substance is in a form which is unaffected by light. Upon movement of the factor into the primary root, inactivation occurs. It should also be noted that white light inhibits root elongation. The degree of inhibition is related to the duration of exposure. Of particular interest in this connection is the fact that red light with transmission in the range of 6100 to 7500 Å and of suffi-

ciently low intensity as to have little effect on root elongation is equally as effective as white light in the inhibition of lateral root initiation. Clearly, these two processes are separate phenomena, involving different factors which are either sensitive to different wave lengths of illumination or which, at a given wave length, show quite different sensitivities to the intensities tested.

An attempt was made to inhibit lateral root formation by exposure of roots to short periods of light daily in lieu of continuous illumination. A short period of incandescent white light was administered once every 24 hours to roots with attached cotyledons cultured in an agar nutrient medium. The data given in table III are averages from two experiments in which a 100-watt incandescent lamp was used at various distances. Measurements were made on the seventh day of the experiment. It is evident that a short exposure to white light once every 24 hours is effective in in-

TABLE III

RELATED EFFECTS OF DURATION AND INTENSITY OF ILLUMINATION ON ELONGATION AND LATERAL ROOT FORMATION OF EXCISED ROOTS WITH ATTACHED COTYLEDON. DAILY ILLUMINATION WITH UNFILTERED 100-WATT INCANDESCENT LAMP.

Exposure time in minutes	Distance from light	Approximate light intensity	Average root length	Laterals per root
	in.	*fc*	*mm.*	
None (dark)	....	....	112	15.9
1	12	120	90	4.9
4	12	120	89	4.6
8	12	120	90	3.3
1	48	5	90	5.9
4	48	5	90	5.4
8	48	5	91	5.6
1	96	1	104	6.7
4	96	1	104	7.2
8	96	1	103	7.1

hibiting lateral root initiation in these roots. The total illumination is considerably less in these treatments than in those using continuous fluorescent illumination. The increased effectiveness of incandescent light is presumably due to the increased intensities in the red range of the spectrum. Inspection of these results suggests the existence of a roughly reciprocal relationship between the light intensity and the duration of exposure in effectively causing lateral root inhibition.

Using the technique of repeated short light exposures, tests were run to determine the least inhibitory wave lengths. Roots with an attached cotyledon were illuminated one minute daily for six days, using a 100-watt incandescent lamp and gelatin filters of known transmission. As no means were available for the measurement of the absolute energies involved, the relative effectiveness of the different filters was difficult to assess. It was clear in all experiments, however, that red and yellow light are as effective

as white light in causing the inhibition. Blue and green light at approximately the same intensity inhibit lateral root formation much less effectively than the other wave lengths tested. On the basis of such experiments, the blue filter was chosen for safe light in all experimental work.

The effect of light on lateral root initiation in isolated roots

Continuous illumination or repeated exposure to light was necessary to prevent lateral root initiation in roots with an attached cotyledon since there was a continuous movement of root-forming factors from the cotyledon; however, with isolated roots in culture, a single short exposure to light was effective in inhibiting lateral root development. A report has already been made (**17**) on the quantitative response, in terms of lateral roots produced, exhibited by isolated roots which have been decapitated at the end of the first week of growth in nutrient culture. An account of the effect of light on the root-forming capacity of such decapitated roots follows.

TABLE IV

EFFECT OF SINGLE PERIODS OF ILLUMINATION ON LATERAL ROOT FORMATION BY DECAPITATED ROOTS GROWN IN STERILE NUTRIENT MEDIUM. FLUORESCENT LIGHT TREATMENT BEGUN AT TIME OF DECAPITATION.

Minutes of illumination	Average root length	Number of laterals on 10 roots
	mm.	
None (dark)	42.4	47
1	40.3	28
10	39.4	26
100	41.3	14
3000	40.8	14
5760	46.3	10
10080 (continuous)	45.0	5

Root tips excised from pea seeds germinated 48 hours were grown in Petri dishes in nutrient medium in the dark for one week by which time they had attained an average length of about 50 mm. After removal of 10 mm. of the tip, the bases were continued in the same medium in the dark for another week. At this time, 35 to 40 lateral roots were apparent on each 10 root bases in the region of the cut end. Decapitated roots maintained in continuous white fluorescent light produced almost no lateral roots (fig. 2 B). In the study of this light effect, root bases were exposed to single periods of light immediately following decapitation for periods varying from one minute to continuous exposure for seven days. Measurements were made one week after decapitation. The data from a typical experiment are presented in table IV, and the results of several such experiments are summarized in figure 3. The latter shows the relationship between duration of exposure and the inhibition of lateral root formation.

The most effective period for inhibition is the initial period of illumination. Ten minutes of exposure to light of 100 fc intensity causes a 50% inhibition of the lateral root formation which would occur in the dark. Additional periods of exposure to light are progressively less effective in causing inhibition of the process of root initiation. Results of experiments in which, after decapitation, the time of exposure to light was delayed for various periods indicate that the most effective time for illumination by white light is during the 24 hour period immediately following decapitation. Brief illumination 48 hours after decapitation is almost non-inhibitory since the root initiation phase has already occurred. It was also found that red light was equally as effective as unfiltered white light while blue light was

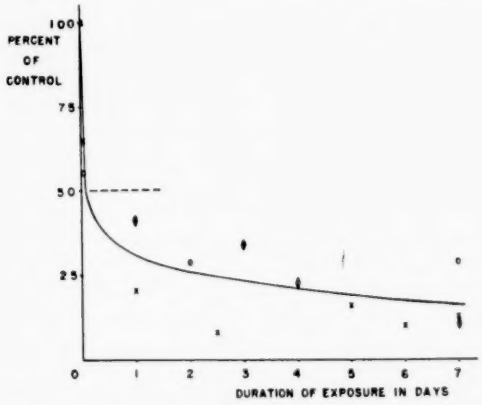

FIG. 3. The effect of duration of exposure to continuous white fluorescent light on lateral root formation by decapitated pea roots. Each point based on an average of 10 roots; data from three experiments included. 100% = dark control.

the least effective in causing inhibition of lateral root formation. The addition of IAA to the nutrient medium (1 mg./l.) following illumination had no effect on lateral root formation.

Discussion

The data presented here give evidence that absorption of radiant energy by pea roots causes inactivation of a substance present within the roots which is necessary for the initiation of cell divisions giving rise to lateral root primordia. In view of the evidence for a light-stimulated oxidation of auxin in plant tissues (**3**), it was thought possible that the inhibition of lateral root initiation was caused by light inactivation of auxin, one of the factors known to be involved in lateral root formation in pea roots; however, the addition of IAA to roots in the dark after illumination had no

effect on lateral root formation. Furthermore, the fact that red light was equally as effective as white light in the inactivation strongly suggests that auxin probably is not the factor directly affected by light. Auxin inactivation *in vitro* by a brei of etiolated pea epicotyls is greatest in the blue-green wave lengths (3900 to 5200 Å), the range of absorption of β-carotene and riboflavin (**4, 18**).

The experimental material should be good subject material for an action spectrum determination. Small amounts of illumination at relatively low intensities cause almost complete inhibition. The number of lateral roots produced is constant for equal total energies, and a reciprocal relationship is evident between the intensity of illumination and the duration of exposure. The results of a number of simple experiments with gelatin filters of known transmission at a given intensity indicate that the most effective wave lengths for inhibition of lateral root formation are within the red range of the spectrum (6100 to 7500 Å) and the least effective are in the blue-green region (4200 to 5400 Å). Although no definite statement concerning the effective wave lengths can be made until a precise determination of an action spectrum has been made, the evidence presented is of interest in view of the numerous recent reports of the spectral sensitivities for photoperiodic control of floral initiation in short and long-day plants (**9, 10**), for leaf and stem growth of etiolated pea seedlings (**11**) and for certain phases of inhibition of growth of the Avena internode (**6**). It is of interest that not only the etiolated and green stem tissues, but the root tissues as well, show morphological responses to illumination of apparently similar spectral distribution. The action spectrum reported by Goodwin and Owens (**6**) for 10% inhibition of internode growth is similar to that given for control of floral initiation (**9, 10**) and is considered an action curve for inhibition of cell division, the same response dealt with in the present experiments. Evidence concerning the nature of the light-absorbing pigment as well as the substances necessary for lateral root initiation may be forthcoming when a more precise knowledge of the action spectrum of this response has been achieved.

Although a detailed analysis of the effect of light on root elongation was not included in this study, some interesting observations on this point are worth noting. The reports in the literature on the influence of light on root elongation are numerous and often conflicting. That no single generalization can be made which will describe the effect of light on root elongation in all species of plants is clear from the review by Hubert and Funke (**7**). The suggestion by Naundorf (**8**) that auxin production in the light determines the root response may lead to an ultimate explanation of the varying responses to white light shown by roots of different species of plants. According to Thimann (**16**) the response of a root to an additional supply of auxin is greatly affected by the auxin concentration already present within the root. The response to auxin has been shown to vary widely from one species to another.

In the experiments reported here, the elongation of isolated roots is markedly inhibited by white light, whereas roots with the shoot attached, but lacking cotyledons, have a rate of elongation equal to those grown in the dark (table I and fig. 1). In this instance auxin production by the shoot may be involved. Since root elongation in low intensities of red light is closely comparable to the elongation of roots grown in the dark, even though lateral root formation is inhibited, it is suggested that auxin concentration in the root may be affected by white light. A careful study of the effect of light on root elongation might contribute to our understanding of the mechanism of root elongation.

Summary

Root elongation and lateral root formation in pea seedlings and isolated pea roots grown in a nutrient medium were studied under various conditions of artificial illumination. In seedlings, removal of the shoot has no effect on lateral root initiation whereas excision of the cotyledons stops lateral root formation under all light conditions. Maximum root elongation and lateral root formation occur when roots are maintained in the dark. White light apparently inactivates substances moving from the cotyledons which are essential for lateral root formation.

Excised roots with an attached cotyledon require continuous illumination or short exposure to light daily to effectively prevent lateral root initiation. Lateral root formation induced in cultured isolated roots by decapitation can be inhibited by a single short exposure to white light. By the use of gelatin filters, it was shown that red light inhibits lateral root formation much more effectively than filtered blue or green light. It is suggested that substances other than auxin are inactivated within the primary root by illumination.

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ABSORPTION AND TRANSLOCATION OF RADIOACTIVE 2,4-DICHLORO-5-IODO^{131}-PHENOXYACETIC ACID BY GREEN PLANTS[1]

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(WITH ONE FIGURE)

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For some years it has been well known that many of the growth promoting substances may, in high concentrations, become very poisonous to some plants but not to others. A number of substituted phenoxy compounds as well as other growth regulators act in this way, but 2,4-dichlorophenoxyacetic acid (2,4-D) has received the greatest attention of investigators. The most important characteristic of this compound is that it is very selective in its action; broadleaved plants are readily injured and even killed by it, whereas the monocotyledonous grasses are injured only by much higher concentrations or not at all. Much use has been made of this selective property in agriculture, and many investigators have attempted to find the answer to the question of why 2,4-D kills the dandelion but not the grass amongst which it grows. There are several approaches open to the investigator and we have chosen to investigate the penetration of the chemical into the plant and its transport to the different parts of the plant.

Until recently quantitative measurements of the absorption of growth regulators, including 2,4-D, has been difficult because of the lack of adequate means of detecting small amounts of the absorbed chemical. Direct measurements of absorption by determination of the residual amounts of 2,4-D remaining on the leaves of treated bean plants have been reported (**5**), and WEINTRAUB *et al.* (**7**) have reported a method of extracting 2,4-D from plants treated with it. More recently the absorption and translocation of growth regulators have been studied by the use of radioactive chemicals. WOOD *et al.* (**8**) studied the absorption of 2-iodo^{131}-3-nitrobenzoic acid by bean and barley plants, and MITCHELL and LINDER (**4**) used 2,4-dichloro-5-iodo^{131}-phenoxyacetic acid with bean plants.

Methods and materials

Plants used in this investigation were: bean, var. Black Valentine; corn, var. White Dent; oats, var. Siegeshafer; wheat, var. Hard Winter White; sunflower, var. Mammoth Russian; dandelion; orchard grass (*Dactylus glomerata*); and the narrow-leaved plantain (*Plantago lanceolata*). All but the last three were grown from seeds in the greenhouse. The last three were dug up in the field and brought into the greenhouse. One to six plants were grown in six-inch pots, depending upon the species. Tungsten illumi-

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nation supplemented normal daylight to give a photoperiod of 15 hours. Temperatures of 65 to 85° F were maintained during the treatment period.

The radioactive growth regulator used was the morpholine salt of 2,4-dichloro-5-iodo^{131}-phenoxyacetic acid (2,4-DI*). The salt was obtained from a commercial source in crystalline form. Solutions for application to the plants and for determination of standards were made by dissolving a known amount of the compound in distilled water or in 95% ethyl alcohol. Application and determination of the absorbed growth regulator were made according to methods outlined by MITCHELL and LINDER (3). A ring of lanolin was stamped on the upper surface of one leaf of each plant under treatment. The ring was placed equidistant from the base and the apex of the leaf blade, confining the applied solution to an area of approximately 0.5 square centimeter.

In most of the experiments each plant received an application of 40 micrograms of the labeled compound (2,4-DI*) contained in 0.02 ml. of solution, applied by a special pipette (3). Tween 20 at the rate of 0.5% was used as a wetting agent. The growth regulator was, in all instances, applied between 6:00 and 8:00 A.M. so that the plants received a long light period after the treatment, since it is known that translocation is aided by light. At the end of the desired period, the leaf to which the 2,4-DI* had been applied was removed; and the rest of the plant was divided into several parts, with like parts from two to six plants constituting a sample. After thorough drying in a forced draft oven, each sample was weighed and ground to pass a 40-mesh screen.

Preparatory to the determination of the activity of the different parts of the treated plants, a 25-mg. aliquot of a sample was evenly distributed on the bottom of a shallow dish made by pressing a brass ring (25 mm. inside diameter) onto the tacky surface of cellophane tape. The dried plant material adhered to the surface of the tape and was essentially one particle in thickness as indicated by the fact that the material adhered to the bottom when the dish was inverted. All samples of an experiment were made ready for activity determination at one time.

Conventional end-window Geiger tube and scaler were used. Counting time was varied depending upon the activity of the sample, the background determinations were made several times during the counting process. Since it was desirable to express the activity of the samples in terms of 2,4-DI* per unit weight of plant tissue, standards were prepared by adding known amounts of 2,4-DI* to 25 mg. of dry tissue from untreated plants, plated on disks in the usual manner. The 2,4-DI* was applied in an alcohol solution which evaporated quickly. Plotting the counts per second against milli-micrograms of 2,4-DI* gave a straight line from which the 2,4-DI* content of the unknown could be determined. Corrections for decay were made when necessary. Experiments were standardized as much as possible with respect to the uniformity of plants and to the point of application of the growth regulator. Although conditions varied somewhat between experi-

ments, in any one experiment all plants were treated in the same way and were subject to the same environmental conditions.

Results

To determine the rate of absorption, corn, wheat, sunflower, beans and oats were used. Each plant was given an application of 40 micrograms of 2,4-DI* within the lanolin ring as previously stated. The plants were harvested at intervals of from 3 to 96 hours after treatment with the labeled growth regulator, and the total amount of 2,4-DI* per plant was determined. In the experiment for which data are given in table I, the bean plants were 18 days old at the time of treatment while the other species were 45 days old. At this stage of development, the primary leaves of the bean were well developed; but the first trifoliate leaves were not yet visible. The oat, wheat and corn plants had four leaves well developed with a fifth immature leaf visible while the sunflower plants bore three pairs of expanded leaves above the cotyledons. Average values for four plants are given as millimicrograms per plant.

TABLE I

AMOUNT OF 2,4-DI* ABSORBED BY VARIOUS SPECIES IN MILLIMICROGRAMS PER PLANT, AT DIFFERENT INTERVALS AFTER TREATMENT, TREATED LEAVES NOT INCLUDED.

Plant	Hours after application					
	3	6	12	24	48	96
Sunflower	0	14	157	382	705	1217
Bean	0	155	226	....	....	1533
Oat	0	17	21	33	36	70
Wheat	0	2	45	87	197	186
Corn	0	0	21	283	243	446

In this experiment the amount of 2,4-DI* present in the treated leaf at the time of harvest was not included in the total. In cases where the treatment extended over only a few hours, considerable amounts of absorbed growth regulator could be expected to be present close to the point of application. To check this possibility experiments were carried out in which the amount of 2,4-DI* present in the treated leaf was determined. At the time the plants were harvested, the leaves receiving the application of 2,4-DI* were removed from the plant and the area bearing the lanolin ring cut out of the leaf by means of a cork borer. This procedure effectively removed all of the 2,4-DI* present on the surface of the leaf. Each leaf was then dried and the 2,4-DI* present in the tissue determined in the usual way. Table II gives the results of these determinations. 2,4-DI* present in the treated leaf is expressed as per cent. of the total found in the whole plant at that time.

From the experiments recorded in tables I and II, it is clear that the 2,4-DI* enters the leaves of the monocotyledonous grasses. However, there

TABLE II

AMOUNT OF 2,4-DI* PRESENT IN THE TREATED LEAF, AS PER CENT. OF THE TOTAL IN THE PLANT AT DIFFERENT INTERVALS AFTER TREATMENT.

Plant	Hours after treatment				
	3	6	12	24	48
Corn	100	100	66	53	24
Bean	100	50	28	....	5
Wheat	100	98	88	86	71
Oat	100	67	68	86	85
Sunflower	100	22	....	16	31

is a great difference between the amounts entering the leaves of the monocotyledons and dicotyledons. The sunflower and bean plants absorbed 1215 and 1533 millimicrograms respectively during a period of 96 hours, whereas the oat, wheat and corn plants absorbed only 70, 186 and 446 millimicrograms during the same period. The movement from the treated leaf was also very slow in the wheat and oats as shown in table II. After 48 hours, 71% of the total amount absorbed by the wheat plant and 85% of that absorbed by the oat plant was still in the treated leaf, whereas only 5% remained in the treated bean leaf. The treated leaves of corn and sunflower retained essentially the same percentage of the applied material. The difference in total amount of 2,4-DI* absorbed and the difference in conduction are very striking and may be involved in the explanation of why monocotyledonous plants are not so readily killed by 2,4-D as are other plants.

Other experiments were conducted in which the absorption was observed over a longer period than 96 hours. In one experiment, the results of which are given in table III, plants of bean, sunflower, corn, wheat, oats, dandelion and orchard grass (*Dactylus glomerata*) were used. The last two species were transplanted from the field and were permitted to establish

TABLE III

ABSORPTION OF 2,4-DI* BY VARIOUS PLANTS. THE TREATED LEAF IS NOT INCLUDED.

Plant	96 Hours			192 Hours		
	Dry weight	2,4-DI*	2,4-DI* / Dry weight	Dry weight	2,4-DI*	2,4-DI* / Dry weight
	mg.	*mμg.*		*mg.*	mμg.	
Bean	578	1533	2.65	737	1571	2.11
Wheat	217	186	0.85	290	175	0.92
Sunflower	1958	1217	0.62	2616	1424	0.54
Dandelion	5025	614	0.12	....	....	....
Orchard Grass	530	120	0.23	....	....	....
Corn	586	446	0.71	708	414	0.44
Oat	329	70	0.21	441	135	0.30

themselves in the greenhouse before being used. The dandelion had immature and partially expanded leaves as well as mature leaves at the time of treatment. The 2,4-DI* was applied to one of the leaves which had fully expanded. Dactylus plants were growing rapidly at the time of treatment with several new leaves developing. Treatment was made to a leaf which was nearly mature. The bean plants were 18 days old, and the sunflower, corn, oat and wheat plants were 45 days old; the beans grow so much faster than the others that when they are 45 days old they are in blossom and may even have fruits. Each plant received an application of 40 gammas of labeled growth regulator on one leaf. This leaf is not included in the values, either for the dry weight or for the total 2,4-DI* absorbed.

In another experiment recorded in table IV, plants were harvested at 72 and 144 hours after application of the growth regulator. In this experiment the plants were younger than those used in the previous experiment. Bean,

TABLE IV

ABSORPTION OF 2,4-DI* BY VARIOUS PLANTS. THE TREATED LEAF IS NOT INCLUDED.

Plant	72 Hours			144 Hours		
	Dry weight	2,4-DI*	2,4-DI* / Dry weight	Dry weight	2,4-DI*	2,4-DI* / Dry weight
	mg.	*mμg.*		*mg.*	*mμg.*	
Bean	491	708	1.43	467	1507	3.23
Sunflower	680	1846	2.71	998	1754	1.76
Corn	131	196	1.49	128	308	2.41
Oat	30	45	1.50	39	212	5.43
Plantain	....	....	....	361	600	1.66
Orchard Grass	....	....	....	366	100	0.27
Dandelion	....	....	....	510	355	0.69

oats and corn were treated when the plants were 12 days old, the sunflowers when 35 days old. Dandelion, orchard grass and narrow-leaved plantain (*Plantago lanceolata*) were obtained from the field but were smaller than those previously described, having only four or five leaves. In these experiments, the dry weight of the plants was also determined to give some point of reference as to the relation between the amount of 2,4-DI* absorbed and the size of the absorbing plant.

When a comparison of the amount of 2,4-DI* per milligram of dry weight is made for the different species, some very odd facts become apparent. In the young oat plants this value is the largest, and in the dandelion it is the lowest. No activity was observed in the large tap root of the dandelion plant, but in the calculation this weight was used. The oat and wheat plants were quite small with only three leaves whereas the sunflower and bean plants were large, and the same amount of 2,4-DI* was applied to each plant. After several hours, so much of the 2,4-DI* had entered the

latter plants that the outside supply was running low, whereas, in the oat and wheat plants it was still very high. This situation would give a greater gradient between the outside and inside of the oat and wheat plants. However, it is quite obvious that 2,4-DI* enters the monocotyledonous plants used in these experiments, yet they are not killed. We must look for some other difference between the two groups of plants. What is the distribution within the plants?

To answer this question, translocation and accumulation of the absorbed growth regulator was studied in several experiments. In beans and sun-

TABLE V

DISTRIBUTION OF ABSORBED 2,4-DI* IN VARIOUS PLANTS. LETTER DESIGNATIONS AS IN FIGURE 1.

Plant	Part	96 Hours		192 Hours	
		Dry weight	2,4-DI*	Dry weight	2,4-DI*
		mg.	*mμg.*	*mg.*	*mμg.*
Sunflower	G	275	360	330	608
	F	326	94	360	428
	E	203	291	358	148
	D	407	344	483	224
	C	427	88	475	16
	B	260	39	310	000
	A	295	000	300	00
Bean	F	43	327	30	340
	E	37	306	67	455
	D	143	506	175	540
	C	131	115	155	52
	B	89	132	90	107
	A	135	57	220	77
Corn	D	154	20	216	49
	C	75	45	67	76
	B	157	305	160	189
	A	300	76	428	100
Wheat	C	73	102	80	91
	B	25	84	40	97
	A	119	00	130	00
Oat	C	82	60	97	35
	B	72	10	74	100
	A	255	00	260	00

flowers the absorbed 2,4-DI* first appeared in the internode below the point of application. High activity was found in the bean hypocotyl six hours after application, while after 12 hours the movement in both species was toward the rapidly growing apical regions. In another experiment, dandelions and plantain showed the greatest accumulation in the small developing leaves of the rosette. The course of translocation and accumulation of absorbed 2,4-DI* is shown in table V.

In the bean and sunflower plants, the absorbed 2,4-DI* rapidly moved toward the young apical part of the plant. To what extent this is influenced

by the transpiration stream cannot be stated; but as already noted, the movement is first into the internode below the node at which the leaf receiving the application is located and only then is there an upward movement. The roots of the sunflower never had any 2,4-DI* in them, and the roots of bean plants had smaller amounts than other parts. In the corn, wheat, and oat plants, the older leaves to which the chemical was applied contained the most 2,4-D. Here the conduction was slow; and during the 192 hours of the experiment, the 2,4-DI* was present in greater amount in the leaf where it was applied or in the part of the stem to which the leaf was attached. There seems to be a block which slows down the transportation

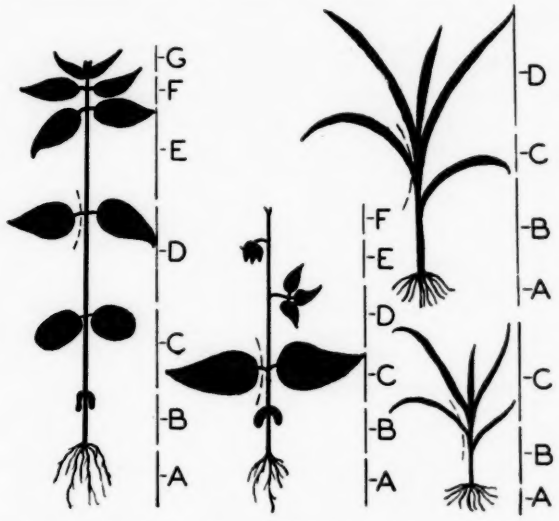

FIG. 1. Diagram showing parts into which plants were separated for analysis. Left, sunflower; center, bean; lower right, oat and wheat; upper right, corn. Broken line indicates treated leaf. See tables V and VI for data.

in the grass plants, whereas in the sunflowers and beans there was a rapid rush of the 2,4-DI* into the young part of the plant.

To determine the extent of redistribution of the 2,4-DI* within the plant the usual type of experiment was carried out with beans and sunflowers. At the end of 96 hours the plants were divided into three lots. One lot was harvested, divided into parts as already indicated and analyzed at once, the second lot had the leaf to which the chemical had been applied removed, and the third lot was left intact as a control. The second and third lots of plants were harvested 96 hours later and the analyses made in the usual way.

There was very litttle absorption during the second 96 hour period (table VI). The outstanding feature of the experiment is that as time

elapsed the 2,4-DI* was moved toward the growing point of the plants to such an extent that in the sunflower the lower half did not have a measurable amount present. In the bean plant, the upward movement was not so complete. These observations indicate that most of the growth regulator did not form a permanent chemical union with any part of the cells. One could hardly consider the data to be evidence that in producing its effect in a plant there is no chemical union of the growth regulator but they do show that the 2,4-DI* is quite mobile and that it moves toward the apex, though

TABLE VI

REDISTRIBUTION OF 2,4-DI* IN MILLIMICROGRAMS IN BEAN AND SUNFLOWER PLANTS, LETTER DESIGNATION AS IN FIGURE 1.

Plant	Part	96 Hours Leaf on plant	192 Hours Leaf not removed	192 Hours Leaf removed after 96 hours
Sunflower	G	360	608	759
	F	94	428	225
	E	291	148	227
	D	344	224	131
	C	88	16	000
	B	39	000	000
	A	000	000	000
	Total	1216	1424	1342
Bean	F	327	340	316
	E	306	455	529
	D	506	540	456
	C	115	52	239
	B	132	107	42
	A	57	77	17
	Total	1443	1571	1599

during the period of active absorption the growth regulator moves both up and down the stem and sometimes even into the roots.

Discussion

The results of the experiments presented here indicate that 2,4-DI* is absorbed by all the species used. The relative amounts of the growth regulator absorbed vary considerably among the species both on the basis of total amount per plant and weight of 2,4-DI* per unit dry weight of plant tissue. The rate of absorption appears to be essentially linear during 96 hours following treatment. Very rapid absorption as reported in the literature (**6**) does not occur under the conditions of these experiments using 2,4-DI*. This observation may be accounted for in part by the fact, as found by MITCHELL and LINDER (**4**) that the morpholine salt of 2,4-DI* is less readily absorbed than is the free acid.

A striking difference between monocotyledonous and dicotyledonous plants appears in the rate at which the absorbed 2,4-DI* is translocated from the treated leaves. Although relatively high concentrations of 2,4-DI* are present in the treated leaves of corn, oat and wheat within a few hours

after application, a very small percentage of the growth regulator is transported to the rest of the plant. During the same period, large amounts of 2,4-DI* are translocated from the treated leaf of sunflower and bean plants. As shown in other experiments reported here, translocation of 2,4-DI* in bean and sunflower plants appears to be to the rapidly growing regions of the plant where there is an accumulation of the chemical. The presence of the intercalary meristem at the base of the monocotyledonous leaf represents a similar growing region and may therefore offer a block to the transport of absorbed 2,4-DI* to the remainder of the plant. EAMES (**1**) has shown for the nutgrass that 2,4-DI* injures or destroys the phloem which would interfere with conduction.

The path of translocation of 2,4-DI* in bean and sunflower plants follows that of the translocation of the products of photosynthesis. This observation is in accordance with the findings of other workers. Movement from the treated leaves is first downward into the internode below the point of treatment. Movement downward may continue, depending upon the species, but the bulk of the growth regulator is later translocated toward the apical parts of the plant. The young rapidly growing regions of the plant appear to be the point of accumulation of absorbed 2,4-DI*.

Summary

A study has been made of the absorption and translocation of the morpholine salt of 2,4-dichloro-5-iodo^{131}-phenoxyacetic acid, (2,4-DI*) by a number of mono- and dicotyledonous plants.

It has been found that this growth regulator is absorbed by all the species used; but that the monocotyledonous plants, oats, wheat and corn absorb it at a slower rate than the others.

The translocation to the apical portion of the plant is very rapid in the bean, sunflower, dandelion, and narrow-leaved plantain, but much less rapid in the grasses. There seems to be a block in the intercalary meristem of the monocotyledonous leaves.

The question of how the compound exerts its selective action on monocotyledonous and dicotyledonous plants has not been answered but the slower absorption and distribution within the monocots are believed to be contributing factors in their greater resistance to 2,4-D.

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CATALASE–CHLOROPHYLL RELATIONSHIP IN BARLEY SEEDLINGS

DAVID APPLEMAN

(WITH TWO FIGURES)

Received December 3, 1951

Introduction

For over two decades H. VON EULER (**3**) and his coworkers have studied the relationship of catalase to chlorophyll. In their work they utilized various chlorophyll defective plants including genetic albinos, variegated, chlorotic and etiolated plants. In general, they found a parallelism between chlorophyll content and catalase activity. NAKAMURA (**11**) working with a number of higher and lower forms of photosynthesizing organisms came to the same conclusions. Our observations on this problem agree in a general way with the above authors with one important exception, namely, the catalase–chlorophyll relationships in etiolated seedlings. EULER and his coworkers (**4**) have reported that catalase activity in etiolated seedlings of barley, rye, wheat and oats are generally lower than in green seedlings. We found that the catalase activity in etiolated seedlings of the above species is consistently and significantly higher than in green seedlings. It is primarily with this phase of the problem that the present paper is concerned.

Material and methods

Barley, variety Sacramento, was used in all the experiments. The details of the method and equipment used for determination of catalase activity are adequately described in a previous publication (**1**). Oxygen liberated from H_2O_2 by the enzyme preparation was measured manometrically. The rate of evolution of oxygen was recorded continuously by means of a simple device. A satisfactory rate measurement was obtained within 30 seconds from the beginning of the reaction.

In the preparation of the samples, only the leaf blades were used. Also, whenever a comparison was made between green and etiolated seedlings, not only were the seedlings grown under exactly the same conditions, with the exception of light, but also corresponding parts of leaves were used. The sampling is important since the catalase activity increases markedly from the base to the tip of the leaf (**5**). A sample (0.5 to 1.5 gm.) of fresh leaves was quickly weighed, on a torsion balance. The leaves were cut with scissors into short sections and placed in a mortar which contained a small, measured, amount of M/10 phosphate buffer, pH 7. The tissue was ground until the suspension was fine enough to be pipetted with a pipette having a large opening. The suspension was transferred to a glass stoppered flask with sufficient buffer solution to make the final suspension contain 10 to 50 mg. of fresh tissue per ml., depending on the expected activity. One ml. of this final suspension was used for catalase determination.

The catalase activities, AF (**1**), are reported as the initial rates AF $= \frac{\text{ml. } O_2 \times 10^3}{\text{mg. of dry sample} \times \text{sec.}}$. Unless otherwise stated, all determinations were made at 0° C and pH 7.

Three other leaf samples similar to the one taken for catalase determination were taken at the same time. One sample was used for determination of per cent. dry matter by drying the sample to constant weight. The second sample was used for determination of total nitrogen by the semimicro Kjeldahl method. The third sample was used for determination of chlorophyll by extracting the leaf sample with methanol and measuring the per cent. transmission of the extract at $\lambda = 6650$ Å on a Beckman spectrophotometer. The specific absorption coefficient (κ) was determined on a purified chlorophyll (a + b) sample and the value, 0.045, was used in all determinations. The chlorophyll is calculated in mg./liter in the extract.

Results

In many preliminary trials performed over a period of several years, we found that the catalase activity in etiolated seedlings of barley, wheat and corn was always higher than in the analogous green seedlings. The etiolated seedlings gave an activity two to ten times as high as the green, depending on age, part of blade used, conditions of culture, and the concentration of H_2O_2 used for the determinations. While the other conditions mentioned generally affect the green and etiolated seedlings in the same direction, beyond a certain point the concentration of H_2O_2 affects the green differently from the etiolated. Figure 1 shows a typical experiment. It is evident that the etiolated plant has its maximum catalase activity at a higher H_2O_2 concentration than the green. To determine whether the difference is due to the enzyme/substrate ratio, the sample of green material used per determination was increased fivefold, to give a rate of oxygen evolution close to that of the etiolated sample. The larger sample, as is evident from the graph, caused no appreciable change in the H_2O_2 concentration at which the maximum activity was obtained. It appears that the catalase in the etiolated seedling may be qualitatively different from that in the green seedling. In order to obtain further support for this possibility, activation energies of the green and etiolated preparations were compared.

Blagoveschenski (**2**) reports that catalases from different plants have different activation energies, as determined by their Arrhenius constants (μ). Owtschinnikow (**13**) found that the μ value of catalase from etiolated wheat seedlings is different from that of the analogous green seedlings. In table I are given some representative Arrhenius constants for barley and wheat catalase which we have determined. Each of the values is an average of three obtained at the three temperature intervals, 0 to 10°, 10 to 20°, 20 to 30° C. The difference between the highest and lowest value within each set never exceeded 600. The values 20 to 30° C showed the greatest variability. In all cases, the etiolated leaves have a lower activation energy

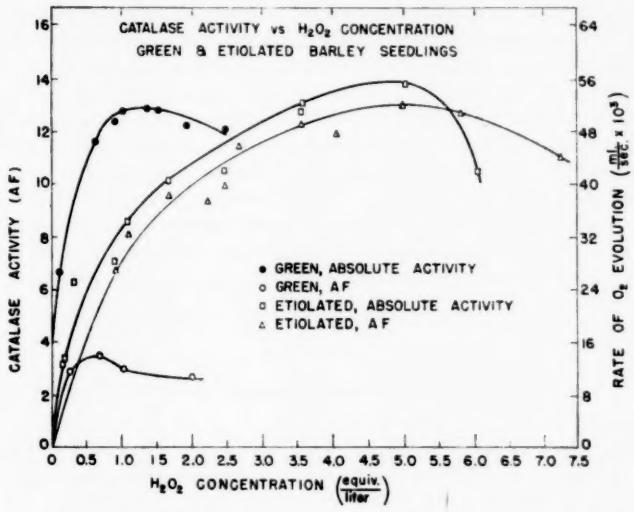
CATALASE ACTIVITY vs H_2O_2 CONCENTRATION
GREEN & ETIOLATED BARLEY SEEDLINGS
CATALASE ACTIVITY (AF)
RATE OF O_2 EVOLUTION ($\frac{ml.}{hr.} \times 10^3$)
H_2O_2 CONCENTRATION ($\frac{equiv.}{liter}$)
• GREEN, ABSOLUTE ACTIVITY
○ GREEN, AF
□ ETIOLATED, ABSOLUTE ACTIVITY
△ ETIOLATED, AF
0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5
0 2 4 6 8 10 12 14 16
0 8 16 24 32 40 48 56 64

FIG. 1. The effect of H_2O_2 concentration on the catalase activity of green and etiolated barley.

than the green. The difference in activation energy is another indication that the catalase in the etiolated seedling may differ qualitatively from that in the green seedling.

The activation energies given in table I were calculated from the Arrhenius equation,

$$\mu = 4.6 \frac{(\log k_2 - \log k_1)}{(1/T_1 - 1/T_2)}$$

where k_1 and k_2 are the rates (AF) at the absolute temperatures T_1 and T_2. The temperatures used were 0, 10, 20, and 30° C. Activation energies are generally given as calories per mole. Since we do not know the actual amount of catalase in our preparations, the values are comparative only.

TABLE I

ACTIVATION ENERGIES (μ) FOR CATALASE IN ETIOLATED AND GREEN SEEDLINGS OF BARLEY AND WHEAT.

Plant	Age in days from planting	Days of exposure to light	μ
Barley	6	0	3020
Barley	6	2	8085
Barley	7	0	5140
Barley	7	4	11330
Barley	10	0	5240
Barley	10	7	11800
Wheat	7	0	3580
Wheat	7	4	9770

Since, upon illumination, an etiolated plant becomes green and acquires all the characteristics of the normally green plant, including its catalase activity, it was of interest to follow the changes in catalase activity during greening. Figure 2 shows the results of such a study. In this experiment, barley seeds were germinated in a dark chamber on screen trays according to the method of HOAGLAND and BROYER (9) in Hoagland solution. On the evening of the third day after planting, when shoots were about 1 cm. long, half the trays were removed from the dark chamber and placed in front of a north window where they remained under normal day and night conditions. The rest were left in the dark chamber. Samples of both sets of plants were taken every evening thereafter for catalase determination. On

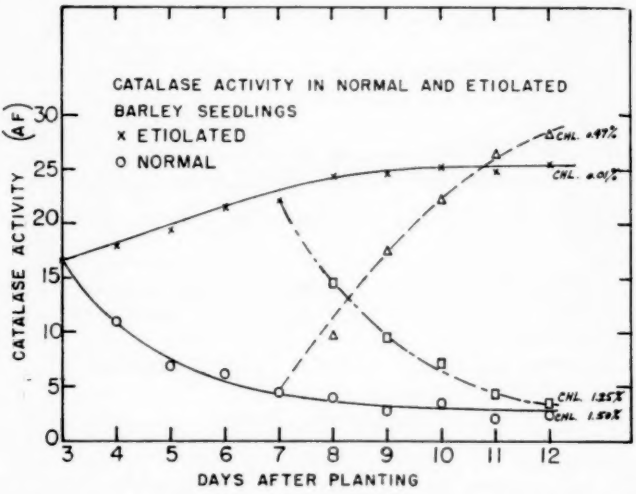

FIG. 2. Changes in catalase activity in barley seedlings induced by light and darkness.

the seventh day, two trays of plants from the dark chamber were moved to the light; and two trays of plants that had been in the light were moved back to the dark chamber.

It is evident from the data that the etiolated plants attained a maximum catalase activity between the eighth and tenth day while at the same time the illuminated plants attained a minimum in catalase activity as well as the normal amount of chlorophyll. An observation not shown on the graph is of interest. Seedlings from the light series which were permitted to continue growth after the twelfth day increased slowly in catalase activity. On the twentieth day they attained a maximum activity of AF 13 which is approximately the value ordinarily found in normally grown barley of this age. In the partially green plants, which were returned to the dark chamber after four days of illumination, catalase activity increased fivefold in

five days of darkness. These plants still retained most of the chlorophyll they accumulated while in the light. When exposed to light the seven-day-old etiolated seedlings lost their catalase activity at a faster rate than the initial, three-day-old seedlings. They also became green more rapidly.

It appears that when chlorophyll is being synthesized rapidly in the plastids, catalase activity decreases; conversely, when chlorophyll synthesis is blocked, catalase activity increases. In order to explore this generalization further, experiments were designed in which chlorophyll synthesis was blocked by means other than etiolation.

LIRO (**10**) in his classic paper has indicated that the terminal process in the chlorophyll synthesis is purely photochemical. The formation of the precursor, however, is markedly influenced by temperature and takes place very slowly if at all, at temperatures below 1° C. SMITH (**15**) has shown conclusively that etiolated seedlings, when illuminated at 1° C, form no more chlorophyll than the amount equivalent to the protochlorophyll pres-

TABLE II

CATALASE ACTIVITY AND CHLOROPHYLL CONTENT IN BARLEY SEEDLINGS GROWN IN THE DARK AT 25°C AND EXPOSED TO ILLUMINATION OF A MAZDA LAMP (250 fc) AT 1 AND 25° C. EXPERIMENTS A AND B WERE DONE AT DIFFERENT TIMES WITH DIFFERENT LOTS OF PLANTS

	Age in days after planting	Days of exposure	Temp. °C	Catalase A F	Chlorophyll as % of dry matter
A	6	0	25	14.0	0.006
	7	1	1	12.8	0.003
	7	1	25	11.0	0.95
B	8	0	25	22.9	trace
	10	2	1	18.9	0.003
	10	2	25	3.70	0.80

ent in them. Presumably, at the low temperature protochlorophyll is not synthesized. We therefore set up an experiment in which chlorophyll formation was blocked by low temperature.

Barley seedlings, grown in the dark for six to eight days at 25° C, were divided into two samples and exposed to light at 25 and 1° C respectively, and the changes in catalase activity were determined. The results, as presented in table II, indicate that when chlorophyll synthesis is blocked, in this case by low temperature, catalase activity suffers only a very small decrease. This observation is not in agreement with EYSTER'S (**6**) belief that when etiolated plants are illuminated the catalase in them is subject to photooxidation. It is possible, however, that in this experiment the light intensity was too low to cause an appreciable inactivation of catalase.

It is well known that certain mineral deficiencies interfere with chlorophyll synthesis. Experience with chlorotic crop plants indicates that they always show a decrease in catalase activity roughly parallel to the decrease in chlorophyll. It was of interest, however, to study the catalase activity

TABLE III

CATALASE ACTIVITY IN THE FIRST LEAF OF BARLEY SEEDLINGS FROM WHICH THE SEED WAS REMOVED AND THE SEEDLINGS GROWN IN SOLUTIONS WITH AND WITHOUT MAGNESIUM.

Age in days after planting	Days on culture solution	Catalase A F	
		+ Mg	− Mg
6	3	8.7	11.8
8	5	7.8	8.4
13	10	6.0	4.8

in the initial stages of a mineral deficiency, before chlorophyll content is reduced. Since Mg is required in relatively high concentration for normal plant growth, we hoped to produce a Mg deficiency in young seedlings. We found, however, that barley seeds have sufficient Mg for the first leaf to develop normally. We resorted to two ways of growing seedlings with a Mg deficiency sufficient to influence chlorophyll content. In the first method, the seeds were germinated on filter paper moistened with distilled water, and the seeds were cut away from the embryos when the coleoptile was about one cm. in length. The seedlings were divided in two lots and planted in Mg-deficient and complete culture solutions. Seedlings, so treated, grew very slowly and were weak and spindly whether grown in a complete or a Mg-deficient medium. In the second method, the barley seeds were germinated and grown in a nutrient solution deficient in Mg. The second leaf was used for catalase determination when it was approximately 10 cm. long.

The results of these experiments are summarized in tables III and IV. These results are interpreted to indicate that just before the concentration of Mg becomes deficient for chlorophyll synthesis but is still sufficient for its other functions, there is a rise in catalase activity. When Mg concentration drops still lower, catalase activity decreases along with chlorophyll content. It would have been desirable to have more frequent sampling. FINKLE (7) working with *Chlorella vulgaris*, cultured in low Mg media, has obtained results which show this phenomenon more strikingly.

Discussion

The experimental observations indicate that etiolated seedlings have a very much higher catalase activity than green seedlings. When etiolated

TABLE IV

CATALASE ACTIVITY AND CHLOROPHYLL CONTENT IN THE SECOND LEAF OF BARLEY SEEDLINGS GROWN IN SOLUTIONS WITH AND WITHOUT MAGNESIUM.

Age in days after planting	Catalase A F		Chlorophyll as % dry matter	
	+ Mg	− Mg	+ Mg	− Mg
12	4.3	8.4	1.39	1.35
14	13.8	12.7	1.61	1.34
16	13.8	7.7	1.53	1.13

seedlings are illuminated, under favorable condition for the synthesis and accumulation of chlorophyll, catalase activity decreases. When etiolated seedlings, high in catalase, are illuminated under conditions in which chlorophyll synthesis does not take place, such as low temperature, there is no appreciable decrease in their catalase activity. When chlorophyll synthesis is blocked by low Mg in the culture medium, there is an increase in catalase activity. Although this increase is of short duration, the fact that it was observed under three different experimental conditions definitely establishes its existence. When green seedlings are placed in the dark their catalase activity increases rapidly. Etiolated grain seedlings have a catalase which is, apparently, qualitatively different from that in the normally green seedlings. While the catalases were not isolated, it is doubtful that the differences observed could be ascribed to substances in the preparation other than catalase. Both the green and etiolated preparations are completely inactivated by heating at 80° C for five minutes or by 10^{-3} molar KCN. Also when a heat inactivated sample of one is added to an active sample of the other, it does not affect the activity in any way. Cell-free extracts of green and etiolated seedlings give results similar to that of the suspensions.

In view of our results, we do not think that a parallelism between chlorophyll and catalase describes the situation adequately. A dynamic equilibrium probably exists between all porphyrin containing substances in the chloroplasts. Under normal conditions a definite quantitative relationship exists between chlorophyll, catalase and probably other substances containing porphyrin. By interfering with the normal functioning of the cell, it is possible to change this quantitative relationship drastically. In the case where chlorophyll synthesis is inhibited by etiolation, the catalase in the etiolated seedlings is more active than in the green, as is indicated by its lower activation energy. Whether this is a general occurrence whenever chlorophyll synthesis is blocked by changes in environmental conditions has not been investigated. Should it prove to be general, it will be of interest to look for a possible functional relationship of this highly active catalase to the biosynthesis of chlorophyll.

GRANICK (**8**) postulated a scheme of heme and chlorophyll synthesis which assumes a common path until the formation of protoporphyrin 9. From the protoporphyrin the synthesis may proceed along two branches, one leading to the hemes, the other to the chlorophylls. In the normal chloroplast both of these substances are present. It is, however, reasonable to expect to find some mutant plants deficient in chlorophyll in which an enzyme, or enzymes, specifically concerned only with the branch of synthesis involving magnesium are inteferred with. In such a mutant one may expect to find catalase activity as high as or higher than in the normal, green plant even though it may be low in or completely devoid of chlorophyll.

We have examined a large number of albino seedlings for catalase activity and, so far, have found one, an avocado seedling which was completely devoid of chlorophyll but had a catalase activity three times as high as the normally green seedlings. This observation is of particular interest

in view of the fact that eight other albino avocado seedlings had catalase activity only 0.2 to 0.3 as high as that in the normal green seedlings, indicative of an interference in the porphyrin synthesis earlier in the path of synthesis probably before the separation into the two branches. Another case is that of an artificially produced *Chlorella vulgaris* mutant which is very pale green. This mutant was supplied to us by Dr. S. Granick of the Rockefeller Institute for Medical Research under his no. 504. In this organism catalase activity is approximately twice as high as in the normal green parent cell. Further work on this and other mutants is now in progress.

The two major pigments of protoplasm, hemoglobin and chlorophyll, occur in highly specialized structures, the red blood cell and the chloroplast. It may be significant that practically all the catalase activity in the blood and in the green plant cell is localized in the red blood cell (**14**) and chloroplast (**12**) respectively. The close association between chlorophyll and catalase in the plastids, coupled with the possibility of inducing experimentally, changes in their relationship offers a fruitful field of research leading to a better understanding of the biosynthesis and relationships of the porphyrin-containing proteins in nature.

Summary

Catalase activity of barley seedlings grown under several experimental conditions was determined. The catalase activity was higher in etiolated seedlings than in green seedlings. When etiolated seedlings were illuminated catalase activity decreased rapidly. Conversely, when seedlings were transferred from light to darkness there was a rapid increase in catalase activity. Etiolated plants, exposed to light at 1° C lose very little catalase activity, but also do not accumulate chlorophyll. Barley plants in the early stages of Mg deficiency have a higher catalase activity than similar plants grown in a complete culture solution. Catalase of etiolated seedlings has a lower activation energy than the catalase in the green seedlings. Arguments are presented to show that, on theoretical grounds, it may be expected to find genetic albinos high in catalase. Two actual examples are cited in support of the arguments.

It is concluded that there is a dynamic equilibrium between the porphyrin-proteins in the chloroplast. When rapid chlorophyll synthesis takes place, catalase activity suffers a decrease. When chlorophyll synthesis is blocked, catalase activity rises rapidly if it is at a low level, or it does not decrease if it is at a high level.

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BRIEF PAPERS

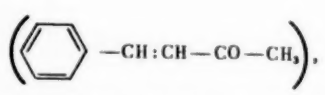
GROWTH REGULATING PROPERTIES OF SOME THIOSEMICARBAZONES

ROBERTSON PRATT, JEAN DUFRENOY AND PETER P. T. SAH

(WITH ONE FIGURE)

Received May 14, 1951

SAH and DANIELS (**6**) reported the synthesis of a large number of thiosemicarbazones which inhibit the growth of *Mycobacterium tuberculosis* var. *hominis* H37Rv. The structure of some of these thiosemicarbazones (table I) is similar in certain respects to the structures of phenylacetic acid and some of its chlorinated oxy derivatives and their hydrazides, of salicylacetone



and of dithiobiuret (NH_2–CS–NH–CS–NH_2), all of which possess pronounced growth regulating properties for some plants (**1, 2, 3, 4**). Therefore, it seemed of scientific as well as of practical interest to test the potential use in agriculture and horticulture of the thiosemicarbazones which are too toxic for practical chemotherapy of tuberculosis. The physical properties of these substances have been reported previously (**6**).

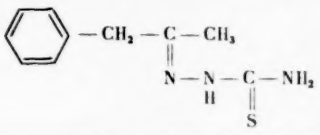
The growth regulating properties of the thiosemicarbazone derivatives listed in table I have been tested on canes of Thompson seedless grapes (*Vitis vinifera*), using a technique described previously (**7**). Briefly, this test involves coating the apical inch of grape cuttings with a paste consisting of Carbowax 1500 in which the drug to be tested is uniformly suspended. Then the cuttings are placed in appropriate vessels with the bases immersed in water or in a solution of potassium nitrate to one and one half or two inches. In the present experiments, each grape cutting consisted of five to six internodes, each cutting received 2 to 2.5 mg. of thiosemicarbazones, and the basal ends of the cuttings were immersed in solutions of KNO_3 that ranged in concentration from 1 gram per liter to 0.0001 gram per liter. For each level of nitrate, the controls consisted of cuttings treated only with Carbowax 1500. All cuttings were dormant when the experiments began. Five to eight cuttings were exposed to each treatment in each experiment.

Most of the thiosemicarbazones tested suppressed in some degree the normal expression of apical dominance in the budding grapevine cuttings. On the control canes the apical buds were the first, and sometimes only, ones to develop leaves. But on treated canes, the thiosemicarbazones frequently

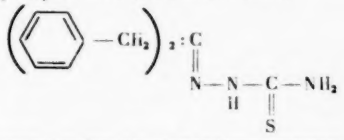
TABLE I

SOME THIOSEMICARBAZONES THAT ARE ACTIVE AGAINST *Mycobacterium tuberculosis* VAR. *hominis* H37Rv AND ALSO EXHIBIT GROWTH-REGULATING ACTIVITY ON CANES OF *Vitis vinifera*.

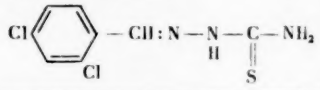
Phenylacetone thiosemicarbazone



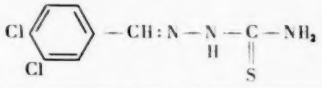
Diphenylacetone thiosemicarbazone



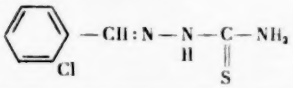
2,4-Dichlorobenzaldehyde thiosemicarbazone



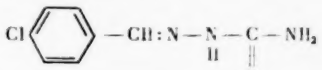
3,4-Dichlorobenzaldehyde thiosemicarbazone



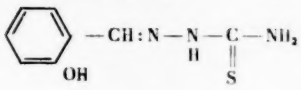
ortho-Chlorobenzaldehyde thiosemicarbazone



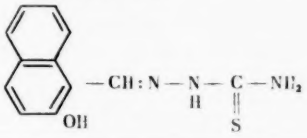
para-Chlorobenzaldehyde thiosemicarbazone



ortho-Hydroxybenzaldehyde (Salicylaldehyde) thiosemicarbazone



2-Hydroxy-1-naphthaldehyde thiosemicarbazone



favored early growth of lower buds at the expense of apical buds. That the apical buds were not killed was evidenced by the fact that they usually grew out later. A quantitative report on this observation will be made after further experimentation.

The principal purpose of this paper is to report another remarkable growth regulating property of four of the thiosemicarbazones, namely, the favoring of early development of flower initials at the expense of leaf development. This effect results in some instances in cuttings that display only small flower buds or at most a minimal number of leafy shoots. Some typical cuttings are shown in figure 1. Four of the thiosemicarbazones were outstanding in enhancing early development of flowers. These were, in decreasing order of efficiency, the thiosemicarbazones of 2,4-dichlorobenzaldehyde, of 3,4-dichlorobenzaldehyde, of 4-chlorobenzaldehyde, and of phenyl acetone. None of these compounds seemed to exert any significant effect on the development of roots by the dormant canes.

In the present experiments, the level of nitrate supplied to the cuttings seemed to have little effect on the response of the cuttings to the compounds tested, especially with regard to the early development of flower buds at the expense of leafy shoots. This fact is in contrast with the responses of simi-

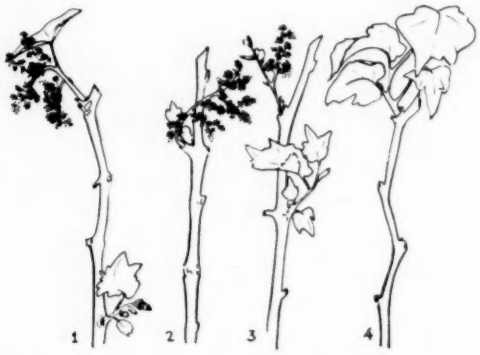


FIG. 1. Typical appearance of cuttings of *Vitis vinifera* (Thompson seedless) 10 weeks after treatment of the dormant canes with some thiosemicarbazones. Only the upper portion of each cane is shown. No. 1 was treated with 3,4-dichlorobenzaldehyde thiosemicarbazone; no. 2 with 2,4-dichlorobenzaldehyde thiosemicarbazone; and no. 3 with phenylacetone thiosemicarbazone. No. 4 is a control with no thiosemicarbazones.

lar cuttings to benzoylhydrazones and other growth regulators, the effects of which on the breaking of dormancy in grape cuttings are greatly influenced by the concentration of nitrate in which the canes are immersed (**5**).

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INCREASING THE SIZE OF THOMPSON SEEDLESS GRAPES BY MEANS OF 4-CHLOROPHENOXYACETIC ACID, BERRY THINNING AND GIRDLING

ROBERT J. WEAVER AND A. J. WINKLER

(WITH ONE FIGURE)

Received December 4, 1951

Girdling has been practiced for years to produce large berries of Thompson Seedless grapes intended for table use. The operation, however, is expensive and occasionally results in the death of the girdled cane. In an effort to discover a better means of attaining results similar to girdling, applications of 4-chlorophenoxyacetic acid have been made (**4, 5**). These tests embraced separate and combined treatments of applications of 4-chlorophenoxyacetic acid, berry thinning (a practice usually employed to overcome compactness of cluster, **6**), girdling, and girdling and berry thinning.

Use was made of an irrigated vineyard located about one mile west of Davis, California. Mature vines of Thompson Seedless were pruned to four or five canes each of which had 9 to 12 buds. Each cane was tied horizontally on one wire of a 3-wire, flat-top trellis. Immediately after fruit set, the crop was reduced to five clusters per cane in tests made in 1950, and six-in tests made in 1951.

In 1950 an oil emulsion of 4-chlorophenoxyacetic acid was prepared, by dissolving 1 gm. of the compound in 5 ml. of an oil-base carrier (**4**), and then diluting to 1000 ml. with distilled water. This stock emulsion was diluted with water to make the desired concentrations. Aqueous solutions of 4-chlorophenoxyacetic acid were prepared in 1951 by adding sufficient ammonia to dissolve the compound. Dreft was used each year as a wetting agent.

Clusters of berries were immersed momentarily into one of the formulations; clusters on one cane constituted a unit in these dipping experiments. Replicate canes were selected at random among the vines used. Berry thinning consisted of cutting off the apical half of the cluster, leaving five or six main branches of the rachis. Girdling consisted of removing a strip of bark three sixteenths of an inch in width near the base of canes with girdling pliers. For the spray applications of the growth regulator, each vine was divided into equal parts; one half was sprayed with a Craftsman sprayer, and the other half served as the untreated control. Cardboards were used to confine the spray to the intended area.

At harvest, berries removed from the same parts of the clusters were thoroughly mixed and the weight of 100 or 200 was obtained in duplicate to indicate the size of the berry. The remaining berries were crushed, and the percentage of total soluble solids in the juice was determined with a Balling hydrometer. Total acidity was determined by diluting 10 ml. of the juice

to 50 ml. with distilled water, and titrating with 0.133 *N* NaOH, using phenolphthalein as an indicator. The results are expressed as grams of acid calculated as tartaric acid per 100 ml. of juice, which is approximately the percentage of acid.

In 1950 treatments were made on June 2 and 7, and in 1951 on June 7. At these times the normal drop of berries following flowering had just occurred. The concentration of growth regulator used for dipping was 20 p.p.m. In 1950 there were five replicate canes per treatment, and 10 in 1951. In 1950 and 1951 the fruit was harvested on September 6 and September 11, respectively. The data (table I) show that clusters treated in 1950 with growth regulator developed larger berries and, with one exception, were heavier than corresponding clusters not treated (fig. 1). Girdling, however, resulted in development of larger berries than did the application of the growth regulator. Clusters dipped in 4-chlorophenoxyacetic acid had thicker pedicels than the clusters of other treatments. In general, the results of 1951 are in accord with those of 1950; clusters treated with growth regulator were larger, and developed larger berries, than corresponding undipped clusters.

TABLE I

AVERAGE WEIGHTS (gm.) PER CLUSTER AND OF 100 BERRIES, PERCENTAGE OF TOTAL SOLUBLE SOLIDS, AND PERCENTAGE OF ACID OF THE JUICE OF THOMPSON SEEDLESS GRAPES.

Treatment	Weight per cluster	Weight of 100 berries	Percentage of total soluble solids	Percentage of acid
1950*				
Untreated control	271.4	127.0	25.3	0.56
Growth regulator only	253.2	161.6	23.5	0.51
Berry-thinned only	227.2	119.7	25.3	0.54
Girdled only	430.9	236.3	24.2	0.56
Growth regulator and berry-thinned	317.7	152.7	25.3	0.52
Growth regulator, berry-thinned, and girdled	563.1	287.9	22.5	0.56
Berry-thinned and girdled	539.3	244.1	23.5	0.57
1951**				
Untreated control	324	146	23.7	0.44
Growth regulator only	483	187	23.4	0.48
Berry-thinned only	260	139	24.4	0.39
Berry-thinned and girdled	612	279	21.1	0.54
Growth regulator, berry-thinned, and girdled	681	327	20.5	0.43

*Significant difference between means for weight of berries at 5% is 41.1 gm. Highly significant difference between means for weight of berries at 1% is 56.6 gm.
**Significant difference between means for weight of berries at 5% is 22.8 gm. Highly significant difference between means for weight of berries at 1% is 30.5 gm.

Spraying was done on June 7, 1951, with the growth regulator at concentrations of 5, 15 or 25 p.p.m. Six replicate vines were used per treatment. The grapes were harvested on September 14. No concentration of the compound used had any apparent effect upon the foliage. In all cases the sprayed clusters produced larger berries than did the controls, and with one

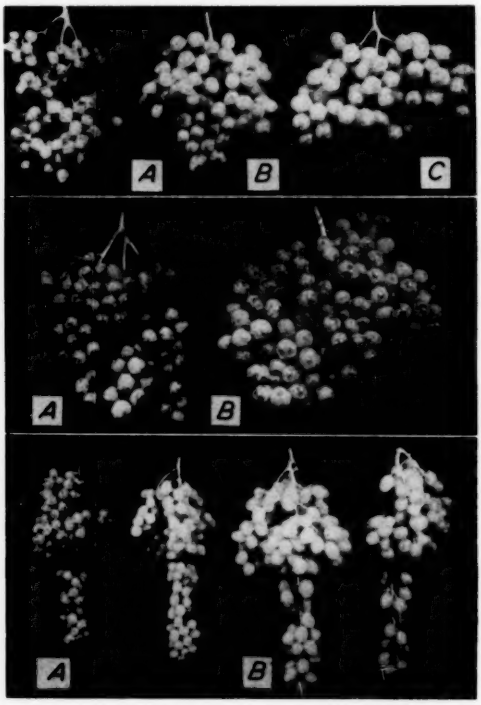

FIG. 1. Effect of treatment with 4-chlorophenoxyacetic acid on the development of Thompson Seedless grapes. Top: A, Berry-thinned. B, Berry-thinned and girdled. C, Dipped in growth regulator at 20 p.p.m., berry-thinned, and girdled. Berries of B and C are larger than those of A and berries of C are largest. Middle: Clusters 91 days after dipping. A, Berry-thinned. B, Dipped in 4-chlorophenoxyacetic acid at 20 p.p.m. and berry-thinned. Berries are larger on cluster treated with the growth regulator. Lower: A, Controls. B, Clusters 99 days after spraying with the growth regulator at 15 p.p.m.; the sprayed berries are larger and have thicker pedicels.

exception the sprayed clusters were larger than the controls (table II, fig. 1). A few clusters on some vines sprayed with the growth regulator at 25 p.p.m. showed some injury. Some berries on these clusters failed to enlarge and the apical one to three inches of the clusters were dead. These clusters were probably very young at the time of treatment. It has been shown that younger clusters of fruit are more easily damaged by growth regulators

TABLE II

AVERAGE WEIGHTS (gm.) PER CLUSTER AND OF 200 BERRIES, PERCENTAGE OF TOTAL SOLUBLE SOLIDS, AND PERCENTAGE OF ACID OF JUICE OF CLUSTERS SPRAYED WITH 4-CHLOROPHENOXYACETIC ACID. EACH FIGURE IS AVERAGE OF SIX REPLICATE VINES.

Concentration of growth regulator (p.p.m.)	Weight per cluster	Weight of 200 berries*	Percentage of total soluble solids	Percentage of acid
0 (untreated half of vine)	56.2	298	23.6	0.46
5 p.p.m.	80.6	368	23.1	0.46
0 (untreated half of vine)	91.9	295	24.2	0.38
15 p.p.m.	79.2	387	24.1	0.39
0 (untreated half of vine)	70.6	316	23.7	0.45
25 p.p.m.	94.8	408	22.2	0.47

*Significant difference between means for weight of berries at 5% is 34.7 gm. Highly significant difference between means for weight of berries at 1% is 47.0 gm.

than are older ones (**4**). However, the few clusters that were injured detracted little, if any, from the total yield of fruit.

Berries on sprayed parts of vines usually had thickened pedicels, which might result in decreased shattering of berries in storage or transit (**2**). Accordingly, a test was made to determine whether the pedicels of berries from vines treated with growth regulator were attached more firmly to the berries than were those of the untreated controls. About 100 berries with pedicels were clipped from each of four vines treated, and the corresponding control lots. The force required to pull each pedicel from its attached berry was then determined with a 1000 gm. scale fitted with an arm for holding a berry while the pedicel was being detached. A gradual pull was exerted until the pedicel broke from the berry. A reading to the nearest 10 gm. was made at the time of severance.

The pedicels of berries from parts of vines sprayed with the growth regulator were more firmly attached, according to average readings, than were pedicels of the corresponding unsprayed berries (table III). Statistical analyses showed these differences between means to be highly significant at 1% in all cases.

TABLE III

EFFECT OF 4-CHLOROPHENOXYACETIC ACID ON THE PEDICEL ATTACHMENT OF THOMPSON SEEDLESS GRAPES. EACH FIGURE IS THE AVERAGE OF 100 READINGS.

Concentration of growth regulator (p.p.m.)	Force (gm.) required to remove pedicels		
	Vine A	Vine B	Average
0 (untreated half of vine)	183	211	197
15 p.p.m.	229	286	258
0 (untreated half of vine)	195	219	207
25 p.p.m.	350	324	337

A test was designed to indicate whether the berries treated with the growth regulator shattered from the clusters less readily than those untreated. One cluster was removed from each lot that had been sprayed with the growth regulator at 15 or 25 p.p.m., and clusters were similarly removed from the corresponding control lots. Ten clusters treated with the compound, and ten clusters untreated, were placed on the laboratory table on September 14, 1951. Five days later each cluster was dropped on its side from a height of about 15 inches. The number of berries that shattered from the treated and untreated clusters was 292 and 387, respectively. At this time the pedicels of both treated and untreated lots had dried, those on the latter clusters often breaking readily near their point of attachment to the branch of the rachis. Pedicels on the treated clusters were usually thicker, tougher, and broke much less readily.

The factors involved in increased strength of attachment of pedicels and decreased amount of berry shatter resulting from application of growth regulator are unknown as yet; increased size of berry (**1, 2, 3**), physiological and nutritional effects, and anatomical changes may all be involved.

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THE EFFECT OF INTERMITTENTLY VARIED PETIOLE TEMPERATURE ON CARBOHYDRATE TRANSLOCATION FROM BEAN LEAVES[1]

WILLIAM A. KENDALL

Received December 21, 1951

The effects of relatively high, intermediate, and low constant petiole temperatures on the translocation of carbohydrates from bean (*Phaseolus vulgaris* var. Black Valentine) leaves of plants otherwise maintained at moderate temperature has been reported by SWANSON and BÖHNING (**1**). The temperature treatments used by these investigators were of considerable duration ranging from 65 to 135 hours. Constant temperature treatments for extended periods of time are not comparable to the highly variable temperature regime of field conditions. It was felt desirable, therefore, to enlarge on these studies by using the approach of these investigators but subjecting the plants to a variable temperature program. Temperature programs were selected which would correspond to: (a) high day temperature and moderately high night temperature, (b) moderately high day temperature and low night temperature, (c) constant moderately high day and night temperature.

The procedure and equipment used in the present investigation were the same as those used by Swanson and Böhning with the exception that only three petiole temperature treatments were used and two of these treatments were varied periodically. The technique employed was to immerse the blade of one primary leaf in a sugar solution, control the petiole temperature, and measure the subsequent elongation of the stem in the dark. This measurement was used as an indirect index of the amount of carbohydrate translocation which had occurred.

In the present investigation three plant units, as described by SWANSON and BÖHNING (**1**), each containing 10 plants, were set up in a dark room with the temperature at $25 \pm 2°$ C. The petioles of the leaves supplied with sugar in one unit were subjected to a temperature of $40 \pm 1.5°$ C for 12 hours and then cooled to $25 \pm 2°$ C for 12 hours in an effort to simulate, except for light conditions, a diurnal temperature cycle consisting of high day temperature and moderately high night temperature. Petioles in the second unit were subjected to a temperature of $25 \pm 2°$ C for 12 hours and then cooled to $5 \pm 5°$ C for 12 hours to correspond to moderately high day temperature and low night temperature. Petioles in the third unit were sub-

[1] Papers from The Department of Botany and Plant Pathology, The Ohio State University, Columbus 10, Ohio, no. 541.

jected to a constant temperature of 25 ± 2° C. The duration of each experiment was 81 hours, during which time three and one half diurnal cycles were completed.

The results of the experiments are presented in table I. The stem elongation values listed in this table are the mean values of seven measurements in experiments 1, 2, and 3, and the mean values of 10 measurements in the remaining experiments. A statistically significant difference between a mean value calculated from measurements on plants exposed to the extreme temperature treatments and the respective value calculated from measurements on plants maintained constantly at 25° C are indicated in the table.

The same general pattern of response was found in experiments 1, 4, 5, and 6. In these experiments the greatest amount of translocation as measured by stem elongation occurred through petioles maintained constantly at 25° C. The smallest amount of translocation occurred through petioles subjected to the 25 and 5° C treatments and an intermediate amount occurred

TABLE I

MEAN STEM ELONGATION AND STANDARD ERROR OF SUGAR TREATED BEAN PLANTS MAINTAINED IN THE DARK AT A TEMPERATURE OF 25°C AND SUBJECTED TO ALTERNATING PETIOLE TEMPERATURES.

Experiment no.	Mean elongation of stems in mm.		
	25° and 50° C	25°C	40° and 25°C
1	6.8 ± 1.6*	31.3 ± 10.9	14.3 ± 5.6
2	23.1 ± 5.2	23.4 ± 4.1	7.6 ± 1.4**
3	12.7 ± 2.5*	23.7 ± 3.9	28.7 ± 3.8
4	17.9 ± 3.3*	55.3 ± 7.8	43.7 ± 13.7
5	16.0 ± 2.2**	42.4 ± 5.7	24.4 ± 3.8**
6	16.5 ± 3.3	25.8 ± 6.4	21.7 ± 3.5

*Significant difference at 5% level of probability.
**Significant difference at 2% level of probability.

through petioles exposed to the 40 and 25° C treatments. In experiment 2 an unusually large amount of stem elongation occurred in plants with petioles subjected to the 25 and 5° C treatments, and in experiment 3 an unusually large amount of elongation occurred in plants exposed to the 40 and 25° C treatments.

The average results of all the experiments in the present investigation are similar to those reported by SWANSON and BÖHNING (1) in that the greatest amount of translocation through petioles occurred at approximately 25° C and at both higher and lower temperatures translocation was retarded. In a comparison of relative translocation values of the two investigations, alternating low and medium petiole temperatures as used in the present studies were found in general to have a greater retarding effect on translocation than the constant low temperatures used by Swanson and Böhning, whereas alternating high and medium temperatures had a lesser retarding effect than constant high temperature.

SWANSON and BÖHNING (1) presented data to show that the retarding effect of low temperature diminished with time, and ascribed this to a physiological re-adjustment to low temperature. Conversely the retarding effect of high temperature in their experiments increased with time perhaps due to progressive injury to the protoplasm. Evidently physiological re-adjustment to low temperatures was slower or less extensive under conditions of intermittent cold as used in these experiments. The greater amount of translocation which occurred when an alternating high and medium temperature was used as compared with a constant high temperature may be due to less injury to the protoplasm as a result of the shorter time of exposure to the very high temperature.

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TEMPERATURE RESPONSE OF FUNGI AS A STRAIGHT LINE TRANSFORMATION

MORRIS COHEN AND C. E. YARWOOD

(WITH THREE FIGURES)

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The extensive data on the relation of temperature to plant pathogens (**15**) and other forms of life are largely empirical. When plotted on arithmetic scales, with temperature as the abscissa and growth as the ordinate, the growth data of many if not all organisms yield a modal type of curve skewed to the right (fig. 1). With data of this type it is difficult to express these temperature relations briefly or to compare one organism with another.

This situation is somewhat similar to that which prevailed in toxicological studies through 1933. The responses of many organisms to known dosages of toxins under standardized conditions had been fairly accurately determined, but these responses could be adequately expressed only by giving the entire mortality data over the dosage series. Then in 1934 BLISS (**1**) showed that mortality data could be expressed as a straight line when

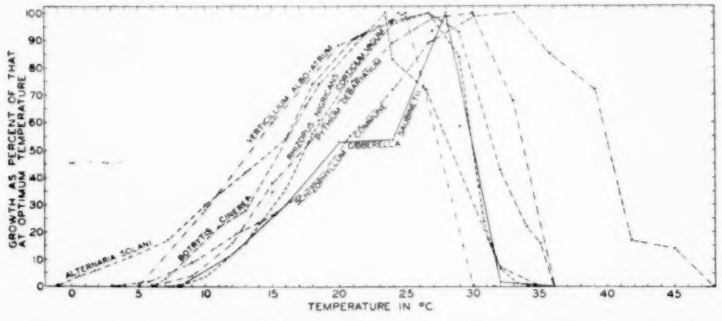

FIG. 1. Temperature-growth data as commonly plotted for *Alternaria solani* (**10**), *Botrytis cinerea* (**9**), *Corticium vagum* (**10**), *Gibberella saubinetii* (**4**), *Pythium debaryanum* (**12**), *Rhizopus nigricans* (**16**), *Schizophyllum commune* (**6**), and *Verticillium albo-atrum* (**5**).

the toxin dosage was plotted on a logarithmic scale against the mortality response on a probit scale. This relation has since been shown to apply to most organisms and toxins which have been studied. When either the LD 95 (or any other LD value) and the slope of the straight line, or two independent LD values are known, an entire set of expected toxicity data for an organism which fits this relation may be derived by interpolation and extrapolation.

The present paper is an initial empirical exploration of the possibility that what has been done to simplify toxicological data may similarly be

done with temperature data, though it is realized that the fundamentals of the two problems are different.

If it is assumed, as shown in figure 1, that temperature-growth response curves for the fungi are basically of the same type, that is, modal and skewed to the right, then their geometric conversion to a straight line relation is readily accomplished. An average curve (fig. 2) may be constructed by averaging horizontally at arbitrary ordinate values the data for the left

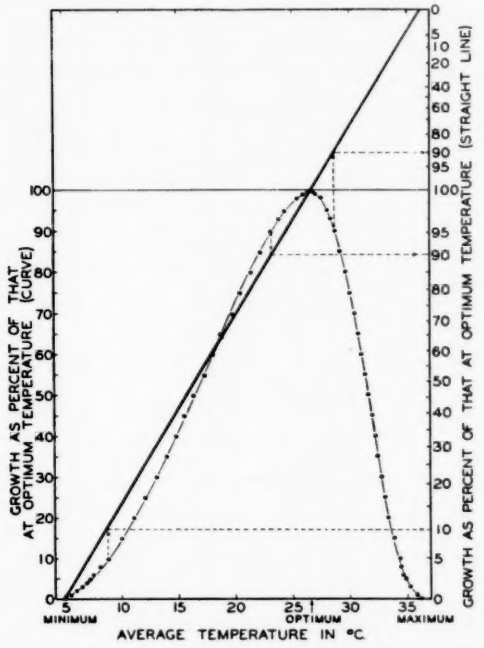
GROWTH AS PERCENT OF THAT AT OPTIMUM TEMPERATURE (CURVE)
GROWTH AS PERCENT OF THAT AT OPTIMUM TEMPERATURE (STRAIGHT LINE)
MINIMUM
OPTIMUM
MAXIMUM
AVERAGE TEMPERATURE IN °C.

FIG. 2. Straight line geometric transformation of average temperature-growth response curve based on data as plotted in figure 1, in addition to data for two strains of *Corticium vagum* (12), two of *Fusarium oxysporum* (8), *Penicillium italicum* (14), *Polyporus vaporarius* (6), another strain of *Pythium debaryanum* (2), and another strain of *Verticillium albo-atrum* (5).

and right hand arms, respectively, of 16 temperature-growth response curves including those shown in figure 1. A straight line is then drawn from the average minimum temperature point (5.0° C, 0%) through the average optimum point (26.8° C, 100%) of the curve and on to its intersection with a vertical erected at the average maximum temperature point (36.2° C, 0%) on the curve.

A new non-linear scale for per cent. of the growth at the optimum is then derived by projecting a series of verticals from points on the curve to points

on the straight line diagonal, and from these intersections a corresponding series of horizontals to their intersection with the right hand ordinate. These last-mentioned intersections are then numbered to correspond with the left hand ordinate values of the points on the curve from which they were respectively derived. In this way the entire growth scale for the temperature range is transformed from an arithmetic scale in which the points above the optimum temperature overlap those below the optimum temperature to one where the points above the optimum temperature are extended beyond the growth at the optimum temperature.

Since the straight line diagonal covers the whole average temperature range for growth, the temperature-growth response relationship of various organisms can, by means of the empirically derived ordinate scale, be represented by straight line diagonals passing through correspondingly various

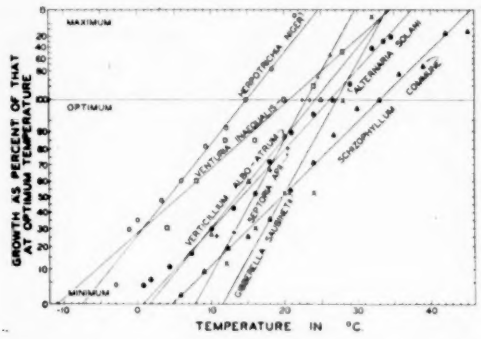

FIG. 3. Extrapolated straight line curves fitted to plotted temperature-growth response data for *Alternaria solani* (**10**), *Gibberella saubinetti* (**4**), *Herpotrichia niger* (**7**), *Schizophyllum commune* (**6**), *Septoria apii* (**3**), *Venturia inaequalis* (**11**), and *Verticillium albo-atrum* (**5**). Original zero growth values are omitted.

optimum temperature points with correspondingly various slopes (various temperature ranges).

Temperature-response data plotted for some of the organisms shown in figure 1, in addition to that for a few others, are plotted on the transformed growth scale as figure 3, the points for zero growth being omitted here.

The advantages of the transformed scale should be apparent. The minimum and maximum temperatures stand out as the upper and lower limits of the graph, and the optimum is clearly defined for each organism. Differences between organisms with respect to minimum, optimum, or maximum temperature or temperature range are clearly apparent. More curves can be arranged in the same space without confusion. Inaccuracies or inconsistencies in collected data are more apparent. If this scale or some similar scale is shown to be applicable to all or most organisms, then it might be possible in new studies to determine experimentally only two points (*e.g.*,

minimum and maximum temperature, or minimum and optimum temperature) and to derive all other values from a graph with a reasonable degree of accuracy.

Two basic weaknesses in the method should be pointed out. First it may be questioned whether the conventional colony diameter measurements of growth as used here represent the true growth, and it may be that if temperature-growth responses were based on dry weight of plant substance, a different curve would result. Not enough temperature-growth responses based on dry weight are available to determine whether the shape of these curves is different from temperature-growth responses based on linear growth. Secondly, most of the data used in this paper were secured in Petri dishes. If this type of container gives a distorted type of temperature-growth curve due to staling and other effects, the transposed straight line scale is correspondingly incorrect. Data for *Neurospora crassa* (**13**) might indicate that if fungus growth were measured in long growth tubes the temperature-growth curve would consist essentially of a straight line ascending to and another descending from the optimum temperature. Not enough temperature-growth data secured from such tubes is available to determine whether this principle applies to most organisms. If these weaknesses prove valid, the empirical scale derived here can be readily corrected to meet these inherent errors.

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EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON PROTEOLYTIC ACTIVITY OF RED KIDNEY BEAN PLANTS[1]

THEODORE L. REBSTOCK, CHARLES L. HAMNER, CHARLES D. BALL AND HAROLD M. SELL

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Recent work has shown that treatment of plants with 2,4-dichlorophenoxyacetic acid (2,4-D) results in a reduction of carbohydrate and an accumulation of nitrogen (**2, 10, 13, 14, 15**). SELL *et al.* (**14**) and WELLER *et al.* (**16**) have observed differences in carbohydrate, nitrogen, and amino acid content that occur primarily in the proliferated stem tissue of the red kidney bean plants. LUECKE *et al.* (**8**) have also noticed changes in the content of several vitamins after treatment with 2,4-D. FELBER (**3**) and NEELY *et al.* (**11, 12**) have shown lower activity for peroxidase, alpha and beta amylase, and phosphorylase, and higher activity for pectin methoxylase in the treated red kidney bean plants. Suggestions have been made that the decrease in carbohydrate and the increase in amino acids and nitrogen content in the stem tissue were due to the conversion in part of the carbohydrates into proteins (**14**). If this postulate is valid then the proteolytic activity might be increased in the treated plants. This communication is a report of the results of an investigation on the effect of 2,4-D on the proteolytic activity of the red kidney bean plants.

Samples of leaf, stem, and root tissue of the red kidney bean plant, *Phaseolus vulgaris*, were prepared according to the procedure previously described by NEELY *et al.* (**12**). The proteolytic activity, using hemoglobin as the substrate, was determined on a sample of one gram of the powdered plant material by using MILLER'S (**9**) revision of the Ayre-Anderson method. Hemoglobin was employed as the substrate since it is a readily available protein and is soluble in solutions at a pH utilized in the analysis. The temperature of the medium was 48° C and a 0.1 M acetate buffer of pH 4.8 was utilized. The amino nitrogen was determined in a Van Slyke amino nitrogen apparatus. For the other proteolytic activity determinations, 0.750 gm. of the appropriate powdered plant material was suspended in 10 ml. of an aqueous 20% glycerol solution for four hours. At 30 minute intervals the reaction tubes were inverted several times to resuspend any settled material at the bottom of the tubes. After four hours the suspension was centrifuged, filtered, and the clear filtrate used as the enzyme preparation. Longer extraction periods did not increase enzymatic activity.

The synthetic substrates consisted of 1% solutions of glycylglycine, L-cystinyldiglycine, L-cystinyldiglycyldiglycine and chloroacetyl-L-tyrosine. The glycylglycine and chloroacetyltyrosine were prepared according to

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FISCHER (4), the L-cystinyldiglycine by the method of LORING and DU VIGNEAUD (7), and the L-cystinyldiglycyldiglycine by the procedure of GREENSTEIN (5).

A modification of the method described by BLAGOWESTSCHENSKI and MELAMED (1) was employed for glycylglycine. For the other synthetic substrates, the peptides were dissolved in distilled water, the pH adjusted to 7 with dilute ammonium hydroxide and distilled water added to give a 1% solution. The hydrolysis was carried out and the amino nitrogen of the substrates, with the exception of L-cystinyldiglycyldiglycine, was determined in a Van Slyke apparatus. The extent of hydrolysis of the latter compound was determined by filtering the reaction mixture and determining the nitrogen in the residue by a semi-micro Kjeldahl procedure.

The results of the various methods with synthetic substrates, hemoglobin, and with different portions of the plant tissue are given in tables I and II. The data in table I were obtained after a study of the effect of temperature and pH indicated maximum hydrolysis during a reaction pe-

TABLE I

EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE PROTEOLYTIC ACTIVITY OF THE STEM, LEAF, AND ROOT TISSUE OF THE RED KIDNEY BEAN PLANT USING A HEMOGLOBIN SUBSTRATE.*

Tissue	Non-treated replicates		Treated replicates	
Stems	1.73	1.89	2.60	2.48
Leaves	1.62	1.93	0.96	0.94
Roots	1.02	1.05	0.88	0.94

*Expressed as the increase in milligrams of amino nitrogen per plant for five hours of reaction at 48° C.

riod of five hours occurred at 48° C and pH 4.8. To be certain that the increase in amino nitrogen was a result of enzymatic hydrolysis of the hemoglobin, separate determinations were made on the substrate and on the plant material. Neither of these determinations indicated a significant increase in amino nitrogen, thus the increase in the amino nitrogen was due to the enzymatic hydrolysis of the hemoglobin substrate. The activity in the stem tissue of the 2,4-D treated plants was almost a third more than in the non-treated stem tissue. A reverse trend was noted in the leaf tissue. The leaves of the non-treated plants showed almost double the proteolytic activity of the treated leaves. The root tissue of the treated plant showed a very slight decrease in activity.

In table II are shown the results in which the proteolytic activity was determined on synthetic peptide substrates. With glycylglycine, the treated stems showed more than double the activity of the control stems; the treated leaves less than half the activity of the non-treated leaves; and the treated roots slightly less activity than the non-treated root tissue. The same trends were observed with cystinyldiglycine as the substrate. The

activity of the treated stem tissue was double the activity of the control tissue; the activity of the treated leaves was less than half that of the non-treated leaves; and the activity of the treated roots was slightly less than the activity of the non-treated roots. Neither cystinyldiglycyldiglycine nor chloroacetyl-L-tyrosine substrates showed evidence of enzymatic hydrolysis when stem, leaf, or root enzyme preparations of red kidney bean plants were employed.

Glycylglycine and cystinyldiglycine are substrates which are utilized for the detection of dipeptidase activity. Chloroacetyltyrosine is a specific substrate for carboxypeptidase, and cystinyldiglycyldiglycine for aminopolypeptidase activity (**5**). Evidently no enzymes are present in the immature bean plants which give carboxypeptidase or aminopolypeptidase activity. Dipeptidase activity was observed in both treated and non-treated tissue. It is of interest that the proteolytic activity determined with hemoglobin, glycylglycine and cystinyldiglycine substrates exhibited the same trends and that the differences between the treated and non-treated tissue were approximately of the same order of magnitude.

TABLE II

EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE PROTEOLYTIC ACTIVITY OF THE TISSUES OF THE RED KIDNEY BEAN PLANT USING SYNTHETIC SUBSTRATES.*

Tissue	Substrate	Non-treated replicates		Treated replicates	
Stem	Glycylglycine	0.22	0.22	0.50	0.45
	L-Cystinyldiglycine	0.54	0.68	1.62	1.18
	L-Cystinyldiglycyldiglycine	0.00	0.00	0.00	0.00
	Chloroacetyl-L-tyrosine	0.00	0.00	0.00	0.00
Leaf	Glycylglycine	0.20	0.21	0.13	0.13
	L-Cystinyldiglycine	0.37	0.28	0.13	0.10
	L-Cystinyldiglycyldiglycine	0.00	0.00	0.00	0.00
	Chloroacetyl-L-tyrosine	0.00	0.00	0.00	0.00
Root	Glycylglycine	0.30	0.20	0.20	0.15
	L-Cystinyldiglycine	0.70	0.82	0.63	0.53
	L-Cystinyldiglycyldiglycine	0.00	0.00	0.00	0.00
	Chloroacetyl-L-tyrosine	0.00	0.00	0.00	0.00

*Expressed as the increase in milligrams of amino nitrogen per plant for 48 hours of reaction at 30° C.

The lower proteolytic activity in the treated leaf tissue was not surprising since there is a slightly lower amino acid content in the treated leaves (**16**). There was also evidence of an inhibition of the growth of the leaf tissue in the treated plants as compared to the controls. The results of the determinations in the stem tissue indicate that there is either an accumulation of protein or free amino acids and other products of protein metabolism in the treated plants. To clarify this problem, the nitrogen components of the stem tissue were partially fractionated, and the nitrogen in each fraction was determined. The results are summarized in table III. The pro-

TABLE III

EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE NITROGEN CONTENT OF THE STEMS OF RED KIDNEY BEAN PLANTS (DRY WEIGHT BASIS).*

	Non-treated replicates		Treated replicates	
	%	%	%	%
Total nitrogen	3.02	3.24	4.61	5.00
Soluble nitrogen (80% ethanol)	1.44	1.55	1.82	2.10
Insoluble nitrogen (80% ethanol)	1.58	1.69	2.79	2.90

*Nitrogen determined by Kjeldahl method.

tein nitrogen was taken to be that portion of the total nitrogen which was insoluble in 80% ethanol while the soluble fraction probably consisted to a large extent of amino acids and other nitrogenous products. The results indicate that the major portion of the increased nitrogen content of the treated stems is in the protein fraction although there is an indication that a small portion of the increase in nitrogen is due to free amino acids or other protein breakdown products.

The difference between the proteolytic activity of the treated and non-treated root tissue was not large enough to be of significance.

These results confirm the postulate indicated by SELL *et al.* (**14**) that one factor associated with the effect of 2,4-D is the utilization of carbohydrate in the synthesis of protein. This increase in protein ($N \times 6.25$) may be responsible for the proliferation of the stems in 2,4-D treated plants. Whether the protein in the treated stems is the same as in the non-treated stems is a problem requiring further investigation. Previous reports have indicated that the amino acids glycine and cystine give high nitrogen values with the Van Slyke method (**6**). The error in the amino nitrogen content reported in this paper because of cystine, and the major portion of the error due to glycine, is eliminated due to the method of determination of proteolytic activity. The error remaining due to glycine is too small to affect the results appreciably.

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PHOTOPERIODIC RESPONSE OF LESPEDEZA[1]

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Received February 8, 1952

Several attempts have been made to establish various leguminous plants as forage and cover crops on pasture land in Hawaii. Korean Lespedeza (*Lespedeza stipulacea* Maxim) and the common Lespedeza (*L. striata* Thunb. H and A) were among those tried. These two species are widely grown throughout the central and southern parts of the United States, where they attain good vegetative growth and are from one to two feet in height. In contrast, when they are grown in Hawaii, these plants attain a height of only a few inches before maturing. Their inability to develop a good vegetative growth in Hawaii suggested that their failure was concerned with the relationship of the plants to the relative length of day. Based on this supposition, a series of experiments was conducted to determine the critical day length for flowering in Lespedeza. SMITH (**2**) reported that Lespedeza can be grown under 14 hours of photoperiod without fruiting. However, the effect of increasing the duration of the light period up to the critical photoperiod for the initiation of flowering has not been investigated.

Four light-tight chambers were constructed. The plants were grown in wooden soil boxes $3 \times 4 \times 1.5$ ft. in size which were placed on galvanized pipe frames fitted with rollers. The boxes were rolled out of the light-tight chambers each morning at 7:30 A.M., and the plants were exposed to normal conditions of sunlight intensity until 4:30 P.M. The length of the various photoperiods was adjusted by exposing the plants in the chambers to additional light from fluorescent lamps regulated by time switches. In each experiment, the treatments were started as soon as the seeds began to germinate. A field test was conducted during the longer days of the year to corroborate the results obtained in the chambers.

Thermographs were set up in each chamber to record diurnal temperature. Comparison of these records with the air temperature records for the locality revealed that the air temperature within the chambers was essentially the same as that of the surrounding area. The daily temperature ranged from 66 to 85° F during the summer months and from 59 to 82° F during the winter months. Air circulation was provided by a light-tight ventilator system in each compartment.

In one experiment, plants of *Lespedeza striata*, started on March 3, 1946, and *Lespedeza stipulacea*, started on April 19, 1946, were subjected to light periods of 10, 12, 14, and 16 hours. Although the number of plants in each treatment was small because of poor germination of seeds, the response of the common Lespedeza to the photoperiods was clear cut. By April 14, 42 days after planting, 100% of the plants exposed to 10 and 12 hours of light

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had mature seeds formed from cleistogamic flowers while the plants exposed to photoperiods of 14 and 16 hours were growing vegetatively without any signs of flowering on June 3. The results obtained with Korean Lespedeza were similar to those of the common Lespedeza. By May 20, 31 days after the seeds were planted, all of the 194 plants in the compartment with 10 hours of light each day had developed mature seeds. Similarly, all of the 102 plants under the photoperiod of 12 hours had seeded; and by June 15, plants receiving 10 and 12 hours of light per day had begun to die. The photoperiods of 14 and 16 hours had not induced flowering by June 3.

The results of the experiments indicated that the critical photoperiod lay between 12 and 14 hours. Hence, the following experiments were designed to determine the longest light period which would initiate the formation of flowers. Korean Lespedeza plants started on September 3, 1946, were used in the first experiment. One set of the plants was left outside of a chamber and exposed to natural day length. For the duration of the experiment the natural day length varied from approximately 12 to 13 hours (3). Three other sets of plants were placed in light chambers, with light periods of 12.5, 13.0, and 13.5 hours, respectively. The plants grown under the natural day length developed cleistogamic flowers, and those grown under photoperiods of 12.5, 13.0, and 13.5 hours developed only petaliferous flowers. On September 28, 25 days after planting, four plants grown under the 12.5 hours of light per day had blossomed; and 19 days later all plants had flowered. The first blossom on plants given 13 hours of light per day was observed on October 4, 32 days after planting, and nearly all plants had flowered by October 30. The first flower on plants growing under the photoperiod of 13.5 hours opened on October 17, and by November 15, 11 plants had open flowers. All of the plants exposed to natural day length had flowered and were begining to die by October 15. Lateral branching was greatest in the plants given 13.5 hours of light per day and poorest in those growing in natural day length.

In the second experiment the plants of Korean Lespedeza were started on November 4, 1946. One set of plants was left under natural day length conditions for a comparison with the plants grown in the controlled photoperiods. The results of this experiment are presented in table I. By December 19, 45 days after planting, all of the 103 plants growing under natural day length had produced seeds from cleistogamic flowers. Twenty-two plants given 13.25 hours of light per day, 13 plants given 13.5 hours of light per day, and one plant given 13.75 hours of light per day had open blossoms on this date. The results of these experiments indicate that the critical photoperiod for flowering is in the neighborhood of 13.75 hours and that photoperiods greater than 14 hours will not result in flowering.

Since the results of tests using the photoperiod chambers indicated that Lespedeza requires a photoperiod of 14 hours or more to maintain vegetative growth without flowering, a field experiment was started during the longer days of the year to determine the growth that can be obtained under

TABLE I

NUMBER OF PLANTS WITH BLOSSOMS UNDER THE VARIOUS PHOTOPERIODS.

Photoperiods	Days after planting						Number of plants with flowers	Total number of plants
	33	42	45	59	68	87		
Natural day length	37	48	18	....	....	....	103	103
13.25 hours	1	7	14	48	16	....	86	86
13.5 hours	1	3	3	23	9	30	75	97
13.75 hours	0	1	0	1	4	10	16	93

field conditions. The day length ranged from approximately 13.75 to 14.5 hours for the duration of the experiment (3). Seeds of five varieties of Lespedeza (Common, Korean F. C. 19604, Korean F. C. 31755, Kobe, and Climax) were planted in field plots one foot wide and four feet long on April 26, 1947. Germination was excellent except in the common variety. The Korean varieties blossomed profusely by the end of May and began to die a month later. These plants grew densely to a height of approximately 11 cm. but branching was meager. The Climax variety performed slightly better with respect to vegetative growth than did the Korean varieties, flowering abundantly three weeks after the Korean varieties. There were three to five lateral branches on most plants, and they grew to a height of 14 cm. before maturing. Plants of the Kobe variety attained the best growth, despite the fact that they grew slowly during the first month. By the end of June, however, these plants attained a height of 15 to 17 cm. and possessed as many branches as the Climax variety. The Kobe variety flowered last, and there were only five plants with flowers at the beginning of July. By the end of July, however, these plants blossomed profusely and attained a height of 19 cm. This field test supports the results of the other experiments. The growth of these species of Lespedeza during the longer days of the year in Hawaii was not comparable to the growth which can be obtained in the continental United States.

The results of these experiments indicate that day length is one of the major factors responsible for the poor vegetative growth of Lespedeza in the Hawaiian Islands. The Korean and common Lespedeza are shown to be short-day plants. When exposed to day lengths of less than 14 hours, these plants initiate flower primordia at the expense of vegetative growth. The effect of lengthening the photoperiod from 13.25 to 13.5 to 13.75 hours was an increasing delay in flowering.

At the latitude of the Hawaiian Islands the day length is too short most of the year to permit Lespedeza to attain good vegetative growth before setting seeds. If day length is measured from sunrise to sunset, as some investigators have done, then at no time does Hawaii have a day length greater than the critical photoperiod of 14 hours (3). However, if the duration of civil twilight (the period after sunset to the time when the

center of the sun is 6° below the horizon) is included, there are two months in which Lespedeza can grow without initiating flower primordia prematurely. To determine whether civil twilight should be included in day length considerations, the intensity of light during the twilight was measured in March and April 1949 with a photometer. Regardless of atmospheric conditions during the day, at the close of civil twilight the light intensity was 1 to 2 fcs. The critical intensity of supplementary light that will prevent the initiation of flower primordia in Lespedeza has not been investigated. According to BORTHWICK and PARKER (**1**), the critical intensity of the supplementary light for Biloxi soybeans is 0.6 fc. WITHROW and BENEDICT (**4**) have indicated that asters and stock require a supplementary light intensity of 0.1 and 10 fcs respectively to bring about the initiation of flower primordia. The requirement for the intensity of supplementary light varies according to the species. The light intensity of 1 to 2 fcs at the close of civil twilight possibly would be sufficient to prevent flowering of Lespedeza. It is evident from these studies that attempts to introduce species of Lespedeza as forage crops on Hawaiian ranch lands will fail unless species that grow well in regions of approximately the same latitude as Hawaii can be found.

Grateful acknowledgment is extended to Mr. Lester Marks, the former Territorial Commissioner of Public Lands (Territory of Hawaii), for material aid in supplying and equipping the experimental chambers, and to the late Dr. Charles J. Engard, for his interest and suggestions during the course of this study. Seeds of the varieties of Lespedeza were obtained through the courtesy of the U. S. Department of Agriculture.

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NOTES

Southern Section.—The Southern Section of the A. S. P. P. met with the Association of Southern Agricultural Workers in Atlanta, Georgia, February 4–6, 1952. A series of papers were presented which were highlighted by a joint symposium with the Horticulture Section entitled "Plant Growth Regulators—Some Fundamental Considerations and Recent Advances in Their Use." Abstracts of all papers will appear in the 1952 Proceedings of the Association. Dr. H. C. Harris gave an address entitled "The Unpredictable Peanut—A Challenge to Scientists" at the traditional breakfast meeting. Both the breakfast meeting and the regular meetings were well attended. The officers elected by the Southern section of the A. S. P. P. for 1952–1953 are: Chairman, HOWARD B. SPRAGUE, Texas Research Foundation; Vice-Chairman, DONALD E. DAVIS, Alabama Polytechnic Institute; Secretary, R. W. BLEDSOE, Florida Agriculture Experiment Station; Directors, JOHN B. WHITNEY, JR., Oak Ridge, Tennessee, E. M. EMMERT, University of Kentucky, G. M. SHEAR, Virginia Agricultural Experiment Station, and PAUL J. KRAMER, Duke University.

Joseph S. Caldwell.—The American Society of Plant Physiologists records with sorrow the passing of one of its members, Joseph S. Caldwell. Dr. Caldwell died on February 18, 1952, at his home in Riverdale, Maryland, after a long illness. Following his retirement from the U. S. Department of Agriculture in 1948, he became a collaborator at the Plant Industry Station and taught food technology at the University of Maryland. He was widely known for the practical value of his researches on the preservation of fruits and vegetables by dehydration and freezing, and on the changes in quality of fruits and vegetables during growth and development. Born near Maryville, Tennessee, October 31, 1878, Dr. Caldwell was a graduate of Maryville College and received his Ph.D. degree from the University of Chicago in 1914. Before entering the Department of Agriculture, he taught at Alabama Polytechnic Institute and also did research in food preservation at the Washington Agricultural Experiment Station.

Modern Trends in Physiology and Biochemistry.—E. S. G. BARRON, Editor. Academic Press, Inc., 125 East 23rd Street, New York 10, New York. 538 pages, 1952. $8.50.

This book is dedicated to the memory of Leonor Michaelis by the physiology department of the Marine Biological Laboratory at Woods Hole. It consists of some of the lectures of the staff in 1950 and some papers contributed by invitation. The topics are: Enzymatic oxidation-reductions; The identification of enzyme-substrate compounds; The cytoplasm, its structure and role; Physiology of the cell nucleus; The physiology of cell

division; Molecular anatomy of the red cell plasma membrane; The measurement of cell permeability; The sodium and potassium balance of muscle and nerve; Bioelectric-potentials; Chemical mechanisms of nerve activity; Transmission processes at nerve-muscle junctions; Ultrastructure and function in nerve; Comparative physiology of nervous systems; Biochemical evolution; Thermodynamics and muscle; The contractile protein system of muscle; The mechanism of drug action; Metal complexes with proteins and other large molecules; The chemical structure and biological activity of chymotrypsin; The oxidative pathways of carbohydrate metabolism. Each chapter has a list of references. Author and subject indexes for the the entire volume are included.

The Chemistry and Technology of Food and Food Products. Volume III.—Morris B. Jacobs, Editor. Second Edition. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 809 pages, 1951. $15.00.

The third volume of this work describes methods of preserving food and the production methods for the principal foods. The types of food preservation given detailed treatment include dehydration, temperature control, hermetically sealed containers, microörganisms, chemicals, and packaging. Production methods are described for cereal grains, bakery products, sugar and sirups, confectionary and cacao products, chewing gum, fruit products, milk products, meat products, fish and shellfish, oils and fats, nonalcoholic beverages, alcoholic beverages, and industrial waters. This volume contains a comprehensive subject index to all three units of the work.

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